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<b>(54) Title:</b> GLYCOSAMINOGLYCAN-DEGRADING ENZYME INHIBITION AND RESULTANT DISEASE THERAPIES			
<b>(57) Abstract</b> <p>Methods for the inhibition of glycosaminoglycan-degrading enzymes are described. These methods involve reacting glycosaminoglycan-degrading enzymes or cells producing the same with an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms. Also described are methods for the treatment of disease associated with glycosaminoglycan-degrading enzymes.</p>			

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## GLYCOSAMINOGLYCAN-DEGRADING ENZYME INHIBITION AND RESULTANT DISEASE THERAPIES

5

### TECHNICAL FIELD

The present invention is directed to methods for the inhibition of glycosaminoglycan-degrading enzymes. The invention is also directed to therapeutic compositions and  
10 methods for the treatment of diseases that involve glycosaminoglycan-degrading enzyme activity.

This invention also relates to sulphur-containing forms of nucleic acid which are capable of inhibiting the activity of enzymes that degrade glycosaminoglycans. In particular,  
15 DNA oligonucleotides containing sulphur-containing backbone linkages are provided which can serve as potent inhibitors of heparin-degrading, and heparan sulphate-degrading enzymes of mammalian and bacterial origin.

20

### BACKGROUND AND RELATED ART

Glycosaminoglycans are linear heteropolysaccharides composed of tandem repeats of a characteristic disaccharide unit (hexosamine-hexuronic acid - see, Kjellen and Lindahl (1991) *Ann. Rev. Biochem.* 60, 443-475). They are synthesized by living cells and are found in intracellular structures, plasma membranes and, in particular, in the  
25 extracellular matrix. The sugar residues within glycosaminoglycans are often decorated with sulphate groups. Glycosaminoglycans can be classified into families based on their sugar composition and the extent and nature of sulphation, and thus a particular glycosaminoglycan may for example be said to belong to one of the heparin, heparan sulphate, chondroitin sulphate, dermatan sulphate or keratan sulphate classes of  
30 glycosaminoglycans. Glycosaminoglycan chains are often linked covalently to protein

molecules to form complex macromolecules known as proteoglycans (Kjellen and Lindahl, 1991). Whether present as free carbohydrate or as part of a proteoglycan, glycosaminoglycans are important structural components of living tissues and in addition are known to exert a powerful influence on the biology of cells in their vicinity,  
5 (Jackson *et al* (1991) *Physiol. Rev.* 71, 481-539 and Ruoslahti (1989) *J. Biol. Chem.* 264, 13369-13372).

Enzymes which degrade glycosaminoglycans play an important role in a number of disease states known to medicine. Glycosaminoglycan-degrading enzymes assist in the  
10 metastatic and invasive processes of cancer by degrading glycosaminoglycan components of the extracellular matrix, and thereby help in the establishment of cancerous cells at secondary sites in the body (Nakajima *et al* (1983) *Science* 220, 611-613 and Nakajima *et al* (1988) *J. Cell. Biochem.* 36, 157-167). They play a part in inflammatory disorders, where, *inter alia*, they assist in cell-mediated immunity by  
15 aiding the extravasation and migration of leukocytes through tissues (Parish (1990) *Today's Life Sci.* Vol 2 No 7, 20-27; Lider *et al* (1990) *Eur. J. Immunol.* 20, 493-499 and Vlodavsky *et al* (1992) *Invasion Metastasis* 12, 112-127). Heparanase activity has also been implicated in the development of some autoimmune disorders, such as experimental autoimmune encephalomyelitis (Naparstek *et al* (1984) *Nature* 310, 241).  
20 Additionally, glycosaminoglycan-degrading enzymes may also be used by pathogens (for example, bacteria) to facilitate invasion of and migration within the tissues of a host organism, in the same way as other tissue-degrading enzymes (for example, proteases) are used for this purpose (Cohen *et al* (1991) *Biochemistry* 30, 11221-11229).

25 Glycosaminoglycans and the enzymes that degrade them additionally play an important role in cardiovascular disease. When atherogenic coronary arteries are unblocked by angioplasty procedures, smooth muscle cells at the site of the lesion often migrate into the arterial lumen and proliferate, (Cascells (1992) *Circulation* 86, 723-729; Bonner (1994) *New Scientist*, No 1931, 32-35). This process, which is known as vascular  
30 hyperplasia, often leads to re-obstruction of the damaged artery in the months following

the angioplasty operation (Liu *et al* (1989) *Circulation* 79, 1374-1387; Bonner, 1994). Such reocclusion is called restenosis, and is most undesirable since it restricts the flow of blood through the artery and may require further surgical intervention (Liu *et al*, 1989; Bonner, 1994; Clowes and Reidy (1991) *J. Vasc. Surg.* 13, 885-891). Since  
5 certain extracellular matrix and cell-surface glycosaminoglycans are thought to play a direct role in inhibiting the change in smooth muscle cell phenotype and in reducing the smooth muscle cell proliferation that characterize vascular hyperplasia, the presence of glycosaminoglycan-degrading activity in the lesion is detrimental (Campbell *et al* (1990) *Ann. N.Y. Acad. Sci.* 598, 159-166; United States No 4,945,086 to Stanford University;  
10 Benitz *et al* (1990) *Am. J. Respir. Cell Mol. Biol.* 2, 13-24; Fritz *et al* (1985) *J. Cell Biol.* 100, 1041-1049; Castellot *et al* (1981) *J. Cell Biol.* 90, 372-379; Castellot *et al* (1987) *Sem. Thrombosis Hemostasis* 13, 489-503; Schmidt *et al* (1992) *J. Biol. Chem.* 267, 19242-19247; Castellot *et al* 1982.). In particular, it has been shown that the degradation of pericellular heparan sulphate glycosaminoglycans by macrophage  
15 heparanase stimulates contractile SMC to change to an altered phenotype associated with cell proliferation and extracellular matrix synthesis (Campbell *et al* (1992) *Exp. Cell Res.* 200, 156-167). Moreover, other research has predicted that platelet heparitinase activity in a restenotic lesion will result in increased SMC growth (Castellot *et al* (1982) *J. Biol. Chem.* 257, 11256-11260). In addition, by analogy to other mechanisms of  
20 cellular invasion through the vessel wall, any degradation of extracellular matrix components in the lesion is likely to assist the migration of smooth muscle cells into the arterial lumen (Parish (1990) *Today's Life Sci.* Vol. 2 No.7, 20-27; Vlodavsky *et al* (1992) *Invasion Metastasis* 12, 112-127; Nakajima *et al* (1983) *Science* 220, 611-613). Exogenously-supplied glycosaminoglycans known to suppress the proliferation of  
25 vascular smooth muscle cells include heparin; modified or non-anticoagulant heparins; and fragments derived from heparin or heparan sulphate (Weissberg (1991) *Ann. Acad. Med.* 20, 38-42; Castellot *et al* (1987) *Sem. Thrombosis Hemostasis* 13, 489-503; WO92/17187 and WO92/17188 to Glycomed; Patent WO90/06755 to Glycomed; EP-394971 to Harvard University and Kabi Vitrum, and Patents WO92/18546 to  
30 Glycomed).

Glycosaminoglycan-degrading enzymes are produced by many mammalian cells and also by certain bacteria. Such enzymes may be endoglycosidases (which cleave glycosaminoglycan chains at internal sites) or exoglycosidases (which degrade glycosaminoglycan chains sequentially from the chain termini). Glycosaminoglycan-degrading enzymes usually exhibit a substrate preference for the type of glycosaminoglycan that they will act upon, and in addition the action of a particular endoglycosidase is usually restricted to cleavage of glycoside bonds joining sugar residues of particular types. Thus, for example, platelet heparanase (heparitinase) is a mammalian  $\beta$ -D-endoglucuronidase which has no strong substrate preference (efficiently degrading both heparin and heparan sulphate) and a specificity for cleavage between glucuronic acid and sulphated N-acetyl glucosamine. Melanoma heparanase has a substrate specificity for heparan sulphate and cleaves between glucuronic acid and sulphated N-acetyl glucosamine, whereas *Flavobacterium heparinum* heparinase I is a bacterial  $\alpha$ -lyase with a substrate preference for heparin and a specificity for cleavage between sulphated glucosamine and sulphated iduronic acid residues (Nakajima *et al* (1984) *J. Biol. Chem.* 259, 2283-2290; Oosta *et al* (1982) *J. Biol. Chem.* 257, 11249-11255; Desai *et al* (1993) *Biochemistry* 32, 8140-8145; Desai *et al*, 1993; Linhardt *et al* (1990) *Biochemistry* 29, 2611-2619). Examples of such enzymes include: mammalian heparanases (EC 3.2.1 - also sometimes called heparitinases) from tumours, including those from melanoma cells, carcinoma cells, sarcoma cells, fibrosarcoma cells, lymphoma cells, myeloma, plasmacytoma, myeloid leukemia cells, and mastocytoma cells; heparanase (heparitinase) from platelets, lymphocytes, endothelial cells, macrophages, neutrophils, and smooth muscle cells; and bacterial heparinase I from *Flavobacterium heparinum* (EC 4.2.2.7), *Flavobacterium heparinum* heparinase II (EC 4.2.2) and *Flavobacterium heparinum* heparinase III (EC 4.2.2.8) and chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris* (Nakajima *et al* 1984; Ricoveri & Cappelletti (1986) *Cancer Res.* 46, 3855-3861; Peretz *et al* (1990) *Int. J. Cancer* 45, 1054-1060; Ricoveri & Cappelletti, 1986 *Supra*; Hennes *et al* (1988) *Br. J. Cancer* 58, 186-188; Laskov *et al* (1991) *Int. J. Cancer* 47, 92-98; Yahalom *et al* (1988) 12, 711-717; Ogren & Lindahl (1975) *J. Biol. Chem.* 250, 2690-2697; Thunberg *et al* (1982)

*J. Biol. Chem.* (1982) 257, 10278-10282; Haimovitz-Friedman *et al* (1991) *Blood* 78, 789-796; Eldor *et al* (1987) *Sem. Thrombosis Hemostasis* 13, 475-488; Oldberg *et al* (1980) *Biochemistry* 19, 5755-5762; Oosta *et al* 1982; Rosenberg (1989) *Meth. Enzymol.* 169, 342-351; Naparstek *et al* (1984) *Nature* 310, 241-244; Laskov *et al* 5 1991; Godder *et al* (1991) *J. Cell. Physiol.* 148, 274-280; JP-1132381; Savion *et al* (1987) *J. Cell. Physiol.* 130, 77-84; Campbell *et al* 1990; Matzner *et al* (1985) *J. Clin. Invest.* 76, 1306-1313; Matzner *et al* (1991) *J. Leukoc. Biol.* 51, 519-524; Desai *et al* 1993; Linhardt *et al* (1990) *Biochemistry* 29, 2611-2617; Lohse & Linhardt (1992) *J. Biol. Chem.* 267, 24347-24355; Lyon & Gallagher (1990) *Anal. Biochem.* 185, 63-70; 10 Linhardt *et al* 1990; Yamagata *et al* (1968) *J. Biol. Chem.* 243, 1523-1535; Oike *et al* (1980) *Biochem. J.* 191, 193-207; and Oike *et al* (1982) *J. Biol. Chem.* 257, 9751-9758.).

Compounds that inhibit the degradation of heparin and heparan sulphate 15 glycosaminoglycans by mammalian heparanases are particularly desirable because of the demonstrated correlation between metastatic potential of tumours and their associated levels of heparanase production, and because of the demonstrated importance of heparanases in inflammation, where they assist blood-borne leukocytes to extravasate and migrate through tissue, as mentioned above (Nakajima *et al* (1984) *J. Cell.* 20 *Biochem.* 36, 157-167; Nakajima *et al* (1986) *Cancer Letts.* 31, 277-283, Lider *et al* (1990) *Eur. J. Immunol.* 20, 493-499; Vlodavsky *et al*, 1992). Compounds that inhibit the degradation of heparin and heparan sulphate glycosaminoglycans by heparanases are also particularly desirable for the treatment of cardiovascular disease as mentioned above because of the demonstrated importance of such glycosaminoglycans in the 25 suppression of smooth muscle cell proliferation in cell cultures of human and other origin and in the suppression of vascular hyperplasia in animal models. The inhibition of smooth muscle cell proliferation achieved by exogenously-supplied glycosaminoglycans (see above) may arise in part from heparanase inhibition by these compounds (Giordano *et al* (1992) *Endothelial Cell Dysfunctions*, Eds. Simionescu and 30 Simionescu, Plenum, New York, pages 49-63; Schmidt and Buddecke (1990) *Europ.*



*J. Cell Biol.* 52, 229-235; Schmidt *et al* (1992) *Europ. J. Cell Biol.* 59, 322-328; Hyslop and Denucci (1993) *Sem. Thrombosis Hemostasis* 19, 89-98; Schmidt *et al* (1992) *J. Biol. Chem.* 267, 19242-19247; Silber *et al* (1993) *Mediat. Inflamm.* 2, 299-302; Benitz *et al* (1990) *Am. J. Respir. Cell Mol. Biol.* 2, 13-24; Bobik and Campbell  
5 (1993) *Pharmacol. Revs.* 45, 1-42; Campbell and Campbell (1986) *Ann. Rev. Physiol.* 48, 295-306; Castellot (1990) *Am. J. Respir. Cell Mol. Biol.* 2, 11-12; Castellot *et al* (1989) *J. Cell Biol.* 109, 3147-3155; Clowes and Reidy (1991) *J. Vasc. Surg.* 13, 885-891; Castellot *et al*, 1987; Edelman *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 3773-3777; Hanke *et al* (1992) *Circulation* 85, 1548-1556; Schmidt and Buddecke (1990)  
10 *Europ. J. Cell Biol.* 52, 229-235; Schmidt *et al* (1992) *Europ. J. Cell Biol.* 59, 322-328; Clowes *et al* (1990) *FASEB J.* 4, A480; Clowes *et al* (1991) *Hypertension* 18, 65-69; Wilson *et al* (1991) *Br. J. Surg.* 78, 1381-1383.)

In view of the importance of glycosaminoglycan degradation in disease states, a number  
15 of attempts have been made to provide inhibitors of glycosaminoglycan-degrading enzymes. In general, it is believed that the endoglycosidases are more important than exoglycosidases in that they commit glycosaminoglycans to sequential degradation by catalyzing the initial cleavages (Nakajima *et al* (1991) *J. Biol. Chem.* 266, 9661-9666). Thus inhibitors of mammalian endoglycosidases are desirable for therapeutic purposes  
20 in medicine. Known inhibitors of mammalian heparanases considered to be useful in these contexts include heparin; modified or non-anticoagulant heparins; sulphated polysaccharides; suramin; and trachyspic acid (Vlodavsky *et al* (1988) *Israel J. Med. Sci.* 24, 464-470; Lider *et al*, 1990); WO92/01003; EP-287477; EP-254067; US 5296471; Vlodavsky *et al*, 1992; Bar-Ner *et al*, (1987) *Blood* 70, 551-557; Irimura *et al* (1986) *Biochemistry* 25, 5322-5328; Parish *et al* (1987) *Int. J. Cancer* 40, 511-518;  
25 Nakajima *et al* (1991) *J. Biol. Chem.* 266, 9661-9666; JP 5086085-A ).

Existing inhibitors of glycosaminoglycan-degrading enzymes suffer from certain shortcomings when considered for use as therapeutic agents in medicine. These include  
30 insufficient potency, insufficient availability, toxicity due to non-specific effects,

imprecise composition, expensive manufacture, and so on. For example, disadvantages can be listed for the heparanase inhibitors mentioned above, as follows. Heparin has an imprecise composition and has potent anticoagulant properties which are often undesirable. Non-anticoagulant derivatives of heparin also have imprecise compositions; moreover, the fact that heparin and heparin derivatives interact with a plethora of extracellular components reduces their availability as heparanase inhibitors such that the low  $IC_{50}$  values obtained *in vitro* (approximately  $0.5\mu M$ , for example, United States Patent No 5,296,471) are unlikely to be achieved *in vivo* (Labat-Robert *et al* (1990) *FEBS Letts.* 268, 386-393). Synthetic oligosaccharides are difficult and expensive to manufacture. Suramin has an  $IC_{50}$  of  $46\mu M$  for melanoma heparanase *in vitro*, which is considered potent, but its efficacy is offset by the fact that this compound is relatively non-specific and inhibits many other enzymes (Nakajima *et al* (1991) *J. Biol. Chem.* 266, 9661-6). Although each of the forgoing examples indicate some success in inhibiting glycosaminoglycan-degrading enzymes, there has been and continues to be a long-felt need for the design of inhibitors which are potent and are capable of effective therapeutic use.

Inhibitors of glycosaminoglycan-degrading enzymes find use as biochemical reagents. Utilizing specific inhibitors, the activities of glycosaminoglycan-degrading enzymes can be assessed and the mechanisms of enzyme activity can be explored. There is a continuing need for such inhibitors.

Modified nucleic acid oligonucleotides, and in particular DNA oligonucleotides synthesized so as to contain phosphorothioate backbone linkages, are routinely used in situations where normal (that is, phosphodiester) DNA would be degraded by cellular nucleases. For this reason the use of phosphorothioate oligonucleotides is widespread in the antisense experiments of gene therapy, where an oligonucleotide complementary to a sequence in target mRNA transcripts is supplied to the cell (with the intention that hybridization of the oligonucleotide to the message should block its translation and/or promote its degradation) (Moffatt (1991) *Science* 253, 510-511). In consequence, the

toxicology and pharmacology of phosphorothioate oligonucleotides have been extensively studied (*Antisense Literature: Antisense Res. Dev.* 1, 65-113 (1991); *Antisense Res. Dev.* 2, 63-107 (1992); *Antisense Res. Dev.* 3, 95-153 (1993)). Phosphorothioate oligonucleotides appear to be well tolerated at micromolar concentrations by cultured mammalian cells and by whole animals. Antisense phosphorothioate oligonucleotides entered clinical trials in human patients several years ago (Wickstrom (1992) *Trends Biotechnol.* 10, 281-287; Zon (1992) *Biotechnol. Intl.*, 119-124; Bayever *et al* (1992) *Antisense Res. Dev.* 2, 109-110). In the course of antisense research, it has come to light that phosphorothioate oligonucleotides can interact with cellular components other than mRNA to cause pleiotropic effects not seen with oligonucleotides made from unmodified DNA. Studies *in vitro* and *in vivo* have shown that phosphorothioate oligonucleotides can inhibit DNA polymerases, RNase H, viral reverse transcriptase and other enzymes that normally act upon nucleic acids (for example, Patents EP-463712-A and WO90/12022-A to University Patents Inc; see also Yaswen *et al* (1993) *Antisense Res. Dev.* 3, 67-77). More surprisingly, however, phosphorothioate oligonucleotides have recently been shown to inhibit HIV replication by binding tightly to the v3 loop of the viral gp120 protein and also to human CD4, whereas their phosphodiester DNA counterparts bind poorly or not at all. (Stein *et al* (1993) *Antisense Res. Dev.* 3, 19-31; Patent WO94/08053 to ISIS Pharmaceuticals; Yakubov *et al* (1993) *J. Biol. Chem.* 268, 18818-18823; Ojwang *et al* (1994) *J. AIDS* 7, 560-570]. In addition, phosphorothioate oligonucleotides (but not their phosphodiester counterparts) are capable of inhibiting phospholipase A<sub>2</sub> (for example, Patent WO 94/08053 to ISIS Pharmaceuticals). The inhibitory effects of phosphorothioate oligonucleotides on certain specific enzymes are unpredictable effects and provide no precedent or expectation that such agents could be used as inhibitors in other enzyme systems.

The present invention arises in part from the surprising finding that oligonucleotides containing at least one backbone linkage substituted with one or more sulphur atoms, such as phosphorothioate and phosphorodithioate oligonucleotides, are potent inhibitors

of glycosaminoglycan-degrading enzymes, and in particular are potent inhibitors of enzymes that degrade sulphated polysaccharides in the course of disease.

## SUMMARY OF THE INVENTION

In one aspect, the invention is directed to a method for the inhibition of glycosaminoglycan-degrading enzymes which comprises reacting said enzymes, or cells producing said enzymes, with an inhibitory effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms.

In a second aspect, the invention is directed to a method for the treatment of disease associated with the glycosaminoglycan-degrading enzyme activity which comprises administering to the subject in need of such treatment a therapeutically effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier.

In another aspect, the invention is directed to a method for the suppression of smooth muscle cell activation, migration and proliferation which comprises contacting said cells with an oligonucleotide as described herein.

In yet another aspect, this invention is directed to a method for the treatment vascular hyperplasia or restenosis which comprises administering to a subject in need of such treatment a therapeutically effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier.

In another aspect, the invention is directed to the use of oligonucleotides for the manufacture of a medicament for the treatment of diseases associated with glycosaminoglycan-degrading enzymes, characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or  
5 more sulphur atoms.

In a still further aspect, the invention is directed to compositions and agents for the treatment of diseases which comprise an oligonucleotide referred to above optionally in association with a pharmaceutically acceptable carrier.

10

In still yet another aspect, the invention is directed to an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, and further characterized in that said oligonucleotide is an inhibitor of glycosaminoglycan-degrading enzymes and/or may be  
15 used in the treatment of disease.

These and other aspects of the invention are described in more detail hereafter.

20

#### DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, biochemistry, protein chemistry and recombinant DNA technology well known to those skilled of the art. Such techniques are explained fully in the relevant literature. For details of the  
25 synthesis and use of oligonucleotides, see, for example, *Oligonucleotide Synthesis*, (M J Gait Ed, 1984, IRL Press, Oxford); *Antisense Res. Dev.* 1, 65-113 (1991); *Antisense Res. Dev.* 2, cc 3-107 (1992); *Antisense Res. Dev.*, 3, 95-153 (1993); Bayeva *et al*, *Antisense Res. Dev.* 2, 109-110 (1992); Zon and Geiser, *Anticancer Drug Des.* 6, 539-568 (1991); and in particular *Oligonucleotides and Analogues: A Practical Approach*,  
30 (Eckstein, Ed, IRL Press, Oxford United Kingdom, 1991).

The following abbreviations and definitions are used throughout this document:

	HS	heparan sulphate
	PCR	polymerase chain reaction
	NaP	sodium phosphate buffer
5	EDTA	ethylenediaminetetraacetate
	BSA	bovine serum albumin
	MES	2-[N-morpholino]ethanesulphonic acid
	PBS	phosphate-buffered saline
	PO	phosphodiester
10	PS	phosphorothioate
	PS <sub>2</sub>	phosphorodithioate
	SMC	smooth muscle cells
	sem	standard error of the mean
	hu	human
15	VSMC	vascular smooth muscle cells
	kDa	kilodaltons
	MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl] tetrazolium bromide)

20 Sequence Identity Numbers (Seq ID Nos) for the nucleotide sequences referred to herein are defined following the examples.

Throughout this document, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the  
25 inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

According to one aspect the present invention is directed to the inhibition of glycosaminoglycan-degrading enzymes which comprises contacting r reacting said  
30 enzymes or cells producing said enzymes with an inhibitory effective amount of an

oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides and the oligonucleotide is substituted with one or more sulphur atoms.

The glycosaminoglycan-degrading enzymes which are inhibited according to this invention are those produced by mammalian cells and microorganisms such as certain bacteria. Mammalian glycosaminoglycan-degrading endoglycosidases which degrade heparin and/or heparan sulphate are referred to as heparanases or heparitinases. For convenience, mammalian heparanases and heparitinases will be collectively referred to as heparanases. Bacterial glycosaminoglycan-degrading endoglycosidases which degrade heparin and/or heparan sulphate are referred to as bacterial heparinases or bacterial heparitinases. Again for convenience, bacterial heparinase and bacterial heparitinase enzymes will be collectively referred to as heparinases. Examples of such enzymes include: mammalian heparanases from tumours, including those from melanoma cells, carcinoma cells, fibrosarcoma cells, lymphoma cells, myeloid leukemia cells, and mastocytoma cells; heparanase from platelets, macrophages, neutrophils, leukocytes, endothelial cells, and smooth muscle cells; and, bacterial heparanases such as heparinase I from *Flavobacterium heparinum*, *Flavobacterium heparinum* heparinase II, *Flavobacterium heparinum* heparinase III, and chondroitinase ABC from *Proteus vulgaris* (Nakajima *et al* 1984; Ricoveri & Cappelletti (1986) *Cancer Res.* 46, 3855-3861; Peretz *et al* (1990) *Int. J. Cancer* 45, 1054-1060; Ricoveri & Cappelletti, 1986 *Supra*; Hennes *et al* (1988) *Br. J. Cancer* 58, 186-188; Laskov *et al* (1991) *Int. J. Cancer* 47, 92-98; Yahalom *et al* (1988) 12, 711-717; Ogren & Lindahl (1975) *J. Biol. Chem.* 250, 2690-2697; Thunberg *et al* (1982) *J. Biol. Chem.* (1982) 257, 10278-10282; Haimovitz-Friedman *et al* (1991) *Blood* 78, 789-796; Eldor *et al* (1987) *Sem. Thrombosis Hemostasis* 13, 475-488; Oldberg *et al* (1980) *Biochemistry* 19, 5755-5762; Oosta *et al* 1982; Rosenberg (1989) *Meth. Enzymol.* 169, 342-351; Naparstek *et al* (1984) *Nature* 310, 241-244; Laskov *et al* 1991; Godder *et al* (1991) *J. Cell. Physiol.* 148, 274-280; JP 1132381; Savion *et al* (1987) *J. Cell. Physiol.* 130, 77-84; Campbell *et al* 1990; Matzner *et al* (1985) *J. Clin. Invest.* 76, 1306-1313; Matzner *et al* (1991) *J. Leukoc. Biol.* 51, 519-524; Desai *et al* (1993); Linhardt *et al* (1990) *Biochemistry*

29, 2611-2617; Lohse & Linhardt (1992) *J. Biol. Chem.* 267, 24347-24355; Lyon & Gallagher (1990) *Anal. Biochem.* 185, 63-70; Linhardt *et al* 1990; Yamagata *et al* (1968) *J. Biol. Chem.* 243, 1523-1535; Oike *et al* (1980) *Biochem. J.* 191, 193-207; and Oike *et al* (1982) *J. Biol. Chem.* 257, 9751-9758.).

5

Glycosaminoglycan-degrading enzymes may be associated with platelets, leukocytes, macrophages, smooth muscle cells and endothelial cells which are intimately associated with the cardiovascular system. For example, these cellular components may be associated with the vessel walls of arteries or veins. This association may arise from the components being an integral structural member of the vessel wall, association through attachment to the vessel wall, association through presence in a plaque, association through presence in the blood, and the like. Glycosaminoglycan-degrading enzymes may also be associated with cancer or tumour cells (for example, being produced by the cell or recruited from other cells such as platelets), or bacterial cells.

15

In general, oligonucleotides comprise a minimum of two nucleotides through to an upper limit of about one hundred nucleotides. This upper limit is notional only in that oligonucleotides of greater number of bases may be employed as long as the oligonucleotides are capable of inhibiting glycosaminoglycan-degrading enzymes. From a practical view point, synthetic procedures for producing oligonucleotides in excess of approximately one hundred nucleotides are less convenient than procedures associated with producing oligonucleotides containing less than about a hundred nucleotides. Longer oligonucleotides (that is, those containing more bases) tend to have a greater inhibitory effect than shorter ones in regard to the inhibition of glycosaminoglycan-degrading enzymes. Preferably the oligonucleotides of this invention comprise seven to thirty bases.

25

Having said this, the only limitations on the number of bases in the oligonucleotide is that the oligonucleotide be capable of inhibiting glycosaminoglycan-degrading enzymes of cardiovascular origin as mentioned above. The term "oligonucleotide" carries its

30



ordinary meaning in the art, that is, a single strand of covalently bonded nucleoside residues wherein the purine or pyrimidine bases and sugar residues are linked by a internucleoside phosphodiester linkage to give a backbone. Oligonucleotides also include oligonucleotides whose base and sugar residues contain modifications, as will  
5 be detailed hereinafter.

The term "oligonucleotide" is generic to polydeoxyribonucleotides (containing 2'-deoxy-ribose or modified forms thereof), that is, DNA; to polyribonucleotides (containing D-ribose or modified forms thereof), that is, RNA; and to any other type of  
10 polynucleotide which is a N-glycoside or a C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base or abasic nucleotides. "Oligonucleotide" also includes mixtures of ribonucleotides and deoxyribonucleotides.

The term "nucleoside" or "nucleotide" is similarly generic to ribonucleosides or  
15 ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbon may be other than that of D-ribose in one or more residues. Also included are analogues where the ribose or deoxyribose moiety is replaced by an alternate structure such as the  
20 6-membered morpholino ring described in United States Patent No 5,034,506 or where an acrylic structure serves as a scaffold that positions the base analogues described herein in a manner that permits efficient binding to target nucleic acid sequences or other targets. As the  $\alpha$ -anomer binds to targets in a manner similar to that for the  $\beta$ -anomers, one or more nucleotides may contain this linkage or a domain thereof  
25 (Praseuth, D *et al*, *Proc. Natl. Acad. Sci.*, (USA) (1988), 85:1349-1353). Modifications in the sugar moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or functionalized as ethers, amines, and the like, are also included.

"Nucleoside" and "nucleotide" include those moieties which contain not only the natively found purine and pyrimidine bases A, T, C, G and U, but also modified or analogous forms thereof. Modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines or other heterocycles. Such "analogous purines" and  
5 "analogous pyrimidines" are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes pseudoisocytosine, N<sup>4</sup>, N<sup>4</sup>-ethanocytosine, 8-hydroxy-N<sup>6</sup>-methyladenine, 4-acetylcytosine,

5-(carboxy- hydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil,

10 5-carboxy-methylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N<sup>6</sup>-isopentenyl-adenine, 1-methyladenine, 1-methylpseudouracil, 1-methyl-guanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N<sup>6</sup>-methyladenine, 7-methylguanine,

15 5-methyl-aminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,

$\beta$ -D-mannosylqueosine,

5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N<sup>6</sup>-isopentenyladenine, uracil-5-oxyacetic acid methylester, pseudouracil, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester,  
20 uracil-5-oxyacetic acid, queosine, 2-thiocytosine, 5-propyluracil, 5-propylcytosine, 5-ethyl-uracil, 5-ethylcytosine, 5-butyluracil, 5-butylcytosine, 5-pentyluracil, 5-pentylcytosine, 2,6-diaminopurine, and 7-deazaguanine.

In addition to the modified bases above, nucleotide residues which are devoid of a  
25 purine or a pyrimidine base may also be included in the oligonucleotides of the invention and in the methods for their obtention.

The sugar residues in the oligonucleotides of the invention may also be other than conventional ribose and deoxyribose residues. In particular, substitution at the 2'-  
30 position of the furanose residue is particularly important.

Oligonucleotides may contain analogous forms of ribose or deoxyribose sugars that are generally known in the art. An exemplary, but not exhaustive list includes 2' substituted sugars such as 2'-O-methyl-, 2'-O-alkyl, 2'-O-allyl, 2'-S-alkyl, 2'-fluoro-2'-halo, or 2'-azido-ribose, carbocyclic sugar analogues,  $\alpha$ -anomeric sugars, epimeric  
5 sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogues and abasic nucleoside analogues such as methyl riboside, ethyl riboside or propyl ribose.

Although the conventional sugars and bases will be used in applying the method of the  
10 invention, substitution of analogous forms of sugars, purines and pyrimidines can be advantageous in designing the final product. Additional techniques, such as methods of synthesis of 2'-modified sugars or carboxylic sugar analogues, are described in Sproat, B D *et al*, *Nucl. Acid Res.* (1991) 19:733-738; Cotten, M *et al*, *Nucl. Acid. Res.* (1991) 19:2629-2635; Hobbs, J *et al*, *Biochemistry* (1973) 12:5138-5145; and  
15 Perbost, M *et al*, *Biochem. Biophys. Res. Comm* (1989) 165:742-747.

The base composition of the oligonucleotides of this invention is not a critical requirement for inhibition to occur since the effect derives from the presence of sulphur in the backbone linkages. The only requirement is that the oligonucleotide is capable  
20 of inhibiting glycosaminoglycan-degrading enzymes of cardiovascular origin. Routine methods for determining the activity of glycosaminoglycan-degrading enzymes of cardiovascular origin are described hereinafter. Having said this, oligonucleotides containing dG and dT tend to inhibit glycosaminoglycan-degrading enzymes to a greater extent than oligonucleotides consisting of dA and dC nucleotides. These findings have  
25 a parallel in the discovery that dG-rich oligonucleotides (and particularly those containing dG<sub>3</sub> and/or dG<sub>4</sub> motifs) constitute the best phosphorothioate ligands for HIV gp120 and make the best phosphorothioate inhibitors of phospholipase A<sub>2</sub> (for example, Patent WO 94/08053 to ISIS Pharmaceuticals). There are other indications in the scientific literature that pleiotropic effects of phosphorothioate oligonucleotides are  
30 enhanced by a high dG content or by inclusion of dG<sub>3</sub> and/or dG<sub>4</sub> motifs (for example,

Yakubov *et al* (1993) *J. Biol. Chem.* 268, 18818-18823). The phosphodiester DNA oligonucleotide of defined sequence ('aptamer') that best inhibits thrombin contains dG<sub>2</sub> motifs that arrange spontaneously into an intramolecular dG-tetrad and ISIS Pharmaceuticals have evidence to suggest that intermolecular dG-tetrads are formed by their dG<sub>3</sub>- and dG<sub>4</sub>-containing phosphorothioate oligonucleotides. (Griffin *et al* (1993) *Gene* 137, 25-31; Padmanabhan *et al* (1993) *J. Biol. Chem.* 268, 17651-17654; Wang *et al* (1993) *Biochemistry* 32, 1899-1904; Wang *et al* (1993) *Biochemistry* 32, 11285-11292; Schultze *et al* (1994) 235, 1532-1547; Patent WO 94/08053 to ISIS Pharmaceuticals.) Phosphodiester DNA aptamers consisting solely of dG and dT nucleotides have been reported to inhibit HIV gp120/CD4 interactions (Ojwang *et al* (1994) *J. AIDS* 7, 560-570).

The oligonucleotides of the invention are characterized in that at least one backbone linkage between adjacent nucleosides is substituted with one or more sulphur atoms. By this is meant that at least one oxygen atom in one or more of the phosphodiester linkages between adjacent nucleosides in the oligonucleotide is replaced with a sulphur atom to give phosphorothioate linkages (replacement of one oxygen atom within a phosphodiester linkage), or phosphorodithioate linkages (replacement of two oxygen atoms within a phosphodiester linkage).

Preferably, each nucleotide in the oligonucleotide chain is linked via a phosphorothio and/or phosphorodithio linkage. Alternatively, an oligonucleotide may comprise a mixture of phosphorothio, phosphorodithio and unsubstituted phosphodiester (phosphate ester) linkages. The precise ratio of unsubstituted linkages to phosphorothioate or phosphorodithioate linkages is not of importance to this invention, as long as the resultant oligonucleotide is capable of inhibiting glycosaminoglycan-degrading enzymes. Experiments carried out by the inventors have shown that oligonucleotides containing only a few phosphorothioate linkages may be effective in inhibiting glycosaminoglycan-degrading enzymes. In general, however, oligonucleotides containing at least 20%,

more preferably at least 50%, still more preferably at least 80%, and most preferably 100% phosphorothioate and/or phosphorodithioate linkages are particularly preferred.

As mentioned previously, the inhibitory potency of phosphorothioate oligonucleotides  
5 increases with oligonucleotide length. This finding has a parallel in the reported length dependence of phosphorothioate oligonucleotides binding to other proteins, for example, HIV gp120 (Stein *et al*, (1993) *Antisense Res. Dev.* 3:19-31).

The preparation of oligonucleotides wherein at least one backbone linkage between  
10 adjacent nucleotides are substituted with one or more sulphur atoms are well established in the art. See Froehler, B *et al*, *Nucl. Acid Res.* (1986) 14:5399-5647; *Nucl. Acid Res.* (1988) 16:4831-4839; *Nucleosides and Nucleotides* (1987) 6:287-291; Froehler, B, *Tet. Let.* (1986) 27:5575-5578 and Zon and Geiser (1991), *Anticancer Drug Des.* 6, 539-568. Oligonucleotides may also be synthesized using solution phase methods such as  
15 triester synthesis, known in the art.

The oligonucleotides of the invention may comprise phosphorothioate or phosphorodithioate diesters. The phosphorothioate diester species may be stereoregular S(p) or R(p) or a mixture of diastereoisomers. The synthesis of stereoregular  
20 phosphorothioate oligonucleotides may be achieved by a number of methods well known to those skilled in the art (Wilk & Stec (1995) *Nucleic Acids Res.* 23, 530-534; Zon & Stec (1991) in *Oligonucleotides and Analogues: A Practical Approach*, Eckstein, Ed, IRL, Oxford, United Kingdom).

25 Oligonucleotides according to the present invention are reacted with or contacted with glycosaminoglycan-degrading enzymes or cells producing the enzymes. This reaction or contact may be a result of directly incubating the oligonucleotides with the enzymes or with cells producing said enzymes. The reaction may take place *in vitro* (such as in an appropriate reaction vessel) or *in vivo*. After application *in vivo*, the inhibitors  
30 should to a large extent be co-localized with their target enzymes. In the absence of

transfection agents, PS oligonucleotides provided to cells remain extracellular or accumulate within endosomes, lysosomes and other vesicles (Tonkinson *et al* (1994) *Antisense Res. Dev.* 4, 269-279; Wagner (1994) *Nature* 372, 333-335; Schick *et al* (1995) *Antisense Res. Dev.* 5, 59-65). For their part heparitinases and heparanases  
5 usually occupy extra-cytoplasmic compartments such as lysosomes, granules, and the extracellular space (Campbell *et al* (1992) *Exp. Cell. Res.* 200, 156-167; Oosta *et al* (1982) *J. Biol. Chem.* 257, 11249-11255; Matzner *et al* (1992) *J. Leukoc. Biol.* 51, 519-524)

10 The amounts of oligonucleotide according to the invention used to inhibit glycosaminoglycan-degrading enzymes will depend on a number of factors including the inhibitory activity of the oligonucleotide, the amount of the glycosaminoglycan-degrading enzyme to be inhibited and specific reaction conditions under which reaction takes place. By way of example only, an effective amount of an oligonucleotide for use  
15 in the inhibition of glycosaminoglycan-degrading enzymes may comprise from 10  $\mu$ g through to 500 mg, or from 1 nmol to 50  $\mu$ mol. It is to be stressed that these examples of inhibitory amounts of oligonucleotides are examples only and do not put any constraints on what constitutes an inhibitory effective amount of an oligonucleotide according to this invention.

20 In accordance with another aspect of this invention, there is provided a method for the treatment of disease associated with glycosaminoglycan-degrading enzymes which comprises administering to a subject in need of such treatment a therapeutically effective amount of an oligonucleotide characterized in that at least one backbone  
25 linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier. The invention further relates to oligonucleotides for use in the treatment of disease associated with glycosaminoglycan-degrading enzymes characterized in that at least one backbone linkage between adjacent nucleosides and the oligonucleotide is  
30 substituted with one or more sulphur atoms.

Diseases associated with glycosaminoglycan-degrading enzymes which may be treated according to this invention include: tumorigenic or neoplastic cell growth (particularly metastasis), such as melanoma, carcinoma, sarcoma, fibrosarcoma, lymphoma, myeloma, plasmacytoma, myeloid leukemia, and mastocytoma; autoimmune disorders associated with heparanase activity, such as autoimmune encephalomyelitis; inflammatory disorders such as arthritis (for example rheumatoid arthritis) and asthma; cardiovascular disease such as vascular hyperplasia, restenosis and atherosclerosis; and infection by pathogenic organisms (particularly pathogenic bacteria). Glycosaminoglycan-degrading enzymes assist in the metastatic and invasive process of cancer by degrading glycosaminoglycan components of the extracellular matrix, thereby helping the establishment of cancer cells at secondary sites in the body (Nakajima *et al* (1983) *Science* 220, 611-613 and Nakajima *et al* (1988) *J. Cell. Biochem.* 36, 157-167). Glycosaminoglycan-degrading enzymes play a part in inflammatory disorders where they assist in cell-mediated immunity by aiding the extravasation of leukocytes (Parish (1990) *Today's Life Sci.* Vol 2 No 7, 20-27; and Vlodavsky *et al* (1992) *Invasion Metastasis* 12, 112-127). Heparanase activity has been implicated in the development of a number of autoimmune disorders, such as experimental autoimmune encephalomyelitis as mentioned above (Naparstek *et al* (1984) *Nature* 310, 241). Cardiovascular disease may result from glycosaminoglycan degradation. Particularly, the degradation of pericellular heparan sulphate glycosaminoglycans by macrophage heparanase stimulates contractile smooth muscle cells to change to an altered phenotype associated with cell proliferation and extracellular matrix synthesis (Campbell *et al*, 1992). Platelet heparitinase activity in a restenotic lesion causes increased smooth muscle cell growth (Castellot *et al*, 1982), and degradation of extracellular matrix is likely to assist in the migration of smooth muscle cells into the arterial lumen (Parish, 1990). These effects cause arterial or vascular occlusion. Additionally, glycosaminoglycan-degrading enzymes may be used by pathogens (for example, bacteria) to facilitate invasion and migration within the tissue of the host organism. The inhibition of glycosaminoglycan-degrading enzymes therefore will assist in the treatment of the aforementioned diseases.

What comprises a therapeutically effective amount of an oligonucleotide in the treatment of disease will depend upon various factors such as the disease being treated, the judgement of the prescribing physician, the activity of the oligonucleotides and the health of the patient being treated. By way of example only, and without limiting the invention, oligonucleotides may be administered to patients in an amount of one nanogram to 500 mg per kilogram body weight.

Diseases associated with glycosaminoglycan-degrading enzymes may be treated by administering oligonucleotides according to this invention parenterally (intravenously, intramuscularly or subcutaneously) orally, rectally or buccally. Oligonucleotides may be directly administered to the site of disease, such as a site of bacterial infection. Suitable formulations are injection solutions, solutions of suspensions for oral therapy, gels, or emulsions. Methods for the therapeutic delivery of oligonucleotides are well known in the art (see, for example, Wickstrom *et al* (1992) *Trends Biotechnol.* 10, 281-287). Oligonucleotides may be incorporated into a matrix that can be deposited locally (that is, for controlled release), for example, by direct injection. Polymer matrices which may be used for controlled release of oligonucleotides include polyesters (polylactine, polyglycolide, polycaprolactone or copolymers), polyalkylcyanoacrylate, polyorthoesters and polyanhydrides. Other biodegradable matrices include various proteins (albumin, gelatin and zein) or polysaccharides (dextrans and starches). Biodegradable materials may be tailored to release their medications continuously or via a timed burst. Thus, the polymer-medication combination can be manipulated to provide desired release properties.

Therapeutic methods for the treatment of cardiovascular disease utilizing oligonucleotides according to this invention generally involve administering the oligonucleotides to the site of cardiovascular trauma. Many methods are known which can achieve this end (Wolensky *et al* (1993), *Trans. Cardiovas. Med.* 3, 163-170). These methods include the use of stents and balloon catheters for local delivery of agents to the arterial wall. Such devices include coated stents, a double balloon with



pores between the respective balloons, a porous balloon, hydrogel coated balloons, a balloon within a porous balloon, or a stented porous balloon. Such stents and catheters are well known and are used in angioplasty procedures. Simons *et al* (1992) *Nature* 359, 667-670, describe gene therapy experiments where antisense phosphorothioate oligonucleotides were delivered to arterial lesions in mammals using adventitial delivery via a pluronic acid gel.

Oligonucleotides delivered to the site of trauma within the cardiovascular system may diffuse into the vessel wall and subsequently act to inhibit glycosaminoglycan-degrading enzymes. In such situations oligonucleotides may be delivered in conventional pharmaceutical vehicles as are well known in the art. Examples of such vehicles include dextrose, ethyl alcohol, and the like.

It may be desirable for oligonucleotide reagents according to this invention to reside within treated vessels for an extended time period after administration via an appropriate catheter. Since inhibition of smooth muscle cell proliferation is a major strategy for the prevention of restenosis, an agent should generally be effective over the time period following injury during which smooth muscle cell proliferation occurs. Oligonucleotides may be incorporated into a matrix that can be deposited locally (that is, for controlled release), for example, by catheter based or surgical techniques. Polymer matrices which may be used for controlled release of oligonucleotides include polyesters (polylactine, polyglycolide, polycaprolactone or copolymers), polyalkylcyanoacrylate, polyorthoesters and polyanhydrides. These may be coupled to an implantable device, such as a stent. Other biodegradable matrices include various proteins (albumin, gelatin and zein) or polysaccharides (dextrans and starches). Biodegradable materials may be tailored to release their medications continuously or via a timed burst. Thus, the polymer-medication combination can be manipulated to provide desired release properties. It is to be appreciated that once the stimulus for starting the cascade of smooth muscle cell proliferation is removed it may be unnecessary for prolonged exposure to inhibitor.

It has been found by the present inventors that phosphorothioate oligonucleotides suppress smooth muscle cell activation, migration and proliferation. The suppression of smooth muscle cell activation blocks the step which is a prerequisite of smooth muscle cell migration and proliferation. Accordingly, in a further aspect this invention is directed to a method for the suppression of smooth muscle cell activation, migration and proliferation which comprises contacting said cells with an oligonucleotide as described herein.

Oligonucleotide compounds as described herein may be used in the treatment of vascular hyperplasia and restenosis. In this aspect, the invention is not limited to the inhibition of glycosaminoglycan-degrading enzymes. The treatment of vascular hyperplasia and restenosis may be additional to or independent of inhibition of glycosaminoglycan-degrading enzymes. By way of example, the suppression of smooth muscle cell activation may not be associated with glycosaminoglycan-degrading enzyme inhibitory activity *per se*.

As previously mentioned, oligonucleotides may be formulated in association with more pharmaceutically acceptable carriers to provide pharmaceutical compositions or agents for the treatment of diseases associated with glycosaminoglycan-degrading enzymes. Preferred pharmaceutical formulations which may be mentioned are solutions, suspensions and emulsions, pastes, tablets, capsules, caplets and suppositories.

Solutions and emulsions can contain, in addition to the active compounds, customary excipients, such as solvents, solubilizing agents and emulsifiers, for example, water, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, glycerol, and the like.

For parenteral administration, the solutions and emulsions can also be in sterile form which is isotonic with blood.

Suspensions can contain, in addition to the active compounds, customary excipients, such as liquid diluents, for example, water, ethyl alcohol or propylene glycol, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose and the like.

5 Orally administrable formulations such as tablets, capsules, pills and granules may contain customary excipients and carriers well known in the pharmaceutical formulation field, and described, for example, in *Remington's Pharmaceutical Sciences*, 10th Edition, Mack Publishing, Philadelphia, USA. For the purpose of convenience, customary excipients, carriers and other agents routinely used and pharmaceutical  
10 formulations may be referred to as "carriers".

In a further aspect of this invention, there is provided a composition for the treatment of disease associated with glycosaminoglycan-degrading enzymes, which composition comprises an oligonucleotide characterized in that at least one backbone linkage between  
15 adjacent nucleotides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier.

Agents for the treatment of diseases associated with glycosaminoglycan-degrading enzymes as mentioned above, comprise oligonucleotide reagents as described herein,  
20 optionally in association with a pharmaceutically acceptable carrier.

In a further embodiment of the invention, there is provided the use of oligonucleotides for the manufacture of a medicament for the treatment of disease associated with the glycosaminoglycan-degrading enzymes, as mentioned above, characterized in that at  
25 least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with at least one or more sulphur atoms. Medicaments may be prepared by simply admixing together modified oligonucleotides as described herein with a pharmaceutically acceptable carrier. This may be achieved by dissolving an oligonucleotide in a liquid carrier, admixing together powdered materials prior to  
30 compounding into tablets or other dosage forms, dissolution into an appropriate solvent,

or other techniques as are well known in the art for production of pharmaceutical formulations.

In a still further aspect this invention is directed to an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, and further characterized in that said oligonucleotide is an inhibitor of glycosaminoglycan-degrading enzymes.

Oligonucleotides according to this invention have the properties as described herein.

Particularly, oligonucleotides may consist wholly or partially of phosphorothioate DNA or phosphorothioate RNA. Alternatively, oligonucleotides may consist wholly or partially of phosphorodithioate DNA or phosphorothioate RNA. In particular, the backbone linkages between adjacent nucleosides in the oligonucleotide may wholly or partially contain phosphorothioate linkages or phosphorodithioate linkages in place of phosphodiester linkages. Particularly preferred are oligonucleotides where all of the backbone linkages between adjacent oligonucleosides are phosphorothioate linkages, or phosphorodithioate linkages. Oligonucleotides may be comprised solely or primarily of dG and/or dT nucleotide. By this is meant that the oligonucleotides contain a base composition of 50% or more dG and/or dT nucleotides, preferably 60% to 100% dG and dT nucleotide. Whilst the number of nucleotides in the oligonucleotide is not critical to the invention, it is preferred that the oligonucleotide comprises from about seven to thirty nucleotides. Particularly preferred oligonucleotides according to this invention have the following sequences:

5'-GGGTTGG-3' (Seq ID No 2),

5'-GGGTTGGTTGTGGGT-3' (Seq ID No 4),

5'-GGGTTGGTTGTGGGTGGGTTGGTTGTGGGT-3' (Seq ID No 6),

5'-GGGGTCGGGGTCGGGGTCGGGGTCGGGGTC-5' (Seq ID No 10).

As exemplified hereafter, SEQ ID No 6 causes significant inhibition (approximately 30%) of human platelet heparanase at 15  $\mu$ M (with heparin as substrate) and at 3  $\mu$ M

(with heparan sulphate as substrate). The oligonucleotide is a 30-mer phosphorothioate oligonucleotide consisting entirely of dG and dT nucleotides.

As stated above, the base composition of the oligonucleotides of this invention is  
5 secondary in importance to the requirement for sulphur-containing backbone linkages. Having said that, the base compositions and sequences of sulphur-containing oligonucleotides can certainly influence and enhance the potency with which they inhibit glycosaminoglycan-degrading enzymes. For example, some sequences may be able to fold into specific three- dimensional structures ('aptamers') that can bind tightly to the  
10 active sites of the enzymes (Bock *et al*, (1992) *Nature* 355, 564-566; Griffin *et al*, (1993) *Gene* 137, 25-31). Thus, considerable improvements in efficacy should easily be attainable for phosphorothioate oligonucleotide inhibitors of glycosaminoglycan-degrading enzymes by utilizing procedures well known in the art to select optimal inhibiting sequences (for example, Larrick (1993) *Gene* 137, 1-3; Stone (1993)  
15 *BioTechnol.* 11, 1508-1509; Alper (1993) *BioTechnol.* 11, 1225; Kenan *et al* (1994) *Trends Biochem. Sci.* 19, 57-64). Iterative affinity-panning approaches depend upon PCR-amplification, which cannot generate phosphorothioate DNA, and hence a positional scanning approach may be used to arrive at optimal inhibitory phosphorothioate sequences (see Example 2 and Bock *et al*, 1992; Griffin *et al*, 1993;  
20 WO 92/14843; WO 91/19813; US 5270163; Ecker *et al*. (1993) *Nucl. Acids Res.* 21, 1853-1856). Alternatively, iterative affinity-panning can be employed to arrive at optimal aptamer sequences consisting of phosphodiester DNA. Since the phosphorothioate counterparts of these aptamers will be more effective inhibitors glycosaminoglycan-degrading enzymes than the phosphodiester forms, even in a  
25 nuclease-free system, such phosphorothioate inhibitors fall within the scope of the present invention. However obtained, optimized sequences should of course be checked for complementarity to known mRNA sequences so as to avoid unwanted antisense effects.

The invention will now be further described with reference to the following non-limiting examples. The invention is exemplified with reference to phosphorothioate oligonucleotides, but is not limited thereto.

5

### EXAMPLE 1

#### Assay for glycosaminoglycan-degrading enzymes

##### Spectrophotometric assays

Since the bacterial heparinases and chondroitinases are lyases which generate  
10 unsaturated products with absorbance at 232 nm, the activity of these enzymes may be  
assayed spectrophotometrically. Assays are carried out according to the procedures of  
Lohse & Linhardt, (1992); and Linhardt *et al* (1990). Enzymes may be purchased from  
the following suppliers: heparinase I and chondroitinase ABC, Seikagaku Corp.  
(Japan); heparinase II, Sigma Chemical Co. (USA). Enzyme units mentioned below  
15 refer to those quoted by the relevant supplier. Heparin is sodium salt Grade 1A from  
Sigma; chondroitin sulphate B is from Seikagaku. Assay mixtures may be made up and  
incubated as follows.

	6 $\mu$ l	Heparinase I, 1 unit/ml 50 mM NaP, pH 7.0
	47 $\mu$ l	BSA, 2mg/ml in 50 mM NaP, pH 7.0
20	5 $\mu$ l	Heparin, 100 mg/ml in water
	100 $\mu$ l	0.5 M NaP, pH 7.0
	100 $\mu$ l	1 M NaCl in water
	5 $\mu$ l	Inhibitor solution in water
	<u>737 <math>\mu</math>l</u>	Water
25	1000 $\mu$ l	Incubate at 30°C
	6 $\mu$ l	Heparinase II, 250 units/ml 50 mM NaP, pH 7.0
	47 $\mu$ l	BSA, 2 mg/ml in 50 mM NaP, pH 7.0
	5 $\mu$ l	Heparin, 100mg/ml in water
30	100 $\mu$ l	0.5 M NaP, pH 7.0

- 5  $\mu$ l Inhibitor solution in water  
837  $\mu$ l Water  
 1000  $\mu$ l Incubate at 30°C
- 5           1  $\mu$ l Chondroitinase ABC, 10 units/ml 50 mM NaP, pH 7.0  
           50  $\mu$ l BSA, 2 mg/ml in 50 mM NaP, pH 7.0  
           100  $\mu$ l Chondroitin sulphate B, 10 mg/ml in PBS  
           30  $\mu$ l 1 M Tris HCl, pH 8.0  
           30  $\mu$ l 1 M Na acetate, pH 7.0
- 10           10  $\mu$ l Inhibitor solution in water  
           779  $\mu$ l Water  
           1000  $\mu$ l Incubate at 37°C
- 15   The bacterial lyases (heparinases I and II, and chondroitinase ABC) were assayed by monitoring at 232 nm the formation of unsaturated products from 40  $\mu$ M heparin or chondroitin-6-sulphate (Nakajima *et al* (1984) *J. Biol. Chem.* 257:11249-11255).
- Polyacrylamide Gel Assays
- 20   Mammalian heparanases are hydrolases which do not generate unsaturated cleavage products, and therefore are not amenable to spectrophotometric assay. Enzymatic activity may however be assessed by monitoring the change in the electrophoretic mobility of the substrate during an incubation with the enzyme. See Gaal *et al* (1989) *Biochem. Biophys. Res. Commun.* 161, 604-614; deVouge *et al* (1994) *Int. J. Cancer*
- 25   56, 286-294; Nakajima *et al*, (1983). Preferably the substrate is a high molecular weight fraction of heparan sulphate or heparin which has been size fractionated by a gel filtration column, for example the excluded-volume fraction from a column of Sephadex G75 Superfine™ (Pharmacia Corporation, Sweden). Uncleaved substrates of this kind migrate slowly towards the anode during electrophoresis in Pharmacia's High Density
- 30   polyacrylamide Phastgels™ (Pharmacia Corporation, Sweden), while the degradation products produced by the action of mammalian heparanases migrate much more rapidly.

The glycosaminoglycan substrates and their cleavage fragments can be visualized by staining with an appropriate stain, preferably Azure A, followed by destaining in running tap-water. Inspection of a stained gel containing different incubation samples run in adjacent lanes enables the effects of enzyme inhibition to be assessed visually (see data at Tables 2 through 4).

For assay of platelet heparanase, a batch of premix solution can advantageously be prepared by mixing:

3  $\mu$ l BSA, 20 mg/ml in 50 mM NaP, pH 7.0  
20  $\mu$ l 0.5 M NaP, pH 6.0  
18  $\mu$ l water  
41  $\mu$ l

For assay of melanoma heparanase, a batch of premix solution can advantageously be prepared by mixing:

3  $\mu$ l BSA, 20 mg/ml in 50 mM NaP, pH 7.0  
3  $\mu$ l 0.54 M D-saccharic acid lactone (Sigma) in water  
20  $\mu$ l 0.5 M NaP, pH 6.0  
15  $\mu$ l 1 M NaCl in water  
41  $\mu$ l

Platelet heparanase is the model glycosaminoglycan-degrading enzyme associated with cardiovascular disease. Human platelet heparanase was obtained as follows. Two sachets of expired platelets were obtained from the Red Cross blood bank, and the platelet-rich serum was centrifuged at 1900 g for twenty minutes at 15°C. White platelet pellets were resuspended in PBS, pH 7.4, at 20°C and re-centrifuged. The white pellets were resuspended in a minimum volume of PBS and freeze-thawed five times using a dry-ice/ethanol bath. The lysate was then centrifuged in an Eppendorf microfuge for twenty minutes at 4°C, and the pelleted debris was discarded. The



supernatant was remicrofuged as above to clarify it further. Clarified supernatant was then passed through a sterile 0.2 $\mu$ m filter to remove contaminants. The resulting lysate is the 'enzyme solution' referred to in the enzyme assay below. Aliquots were snap-frozen and stored at -70°C.

5

Melanoma heparanase was obtained for the purpose of testing the invention as follows. Murine melanoma B16-BL6 cells were grown in culture to three-quarter confluence and harvested by EDTA treatment, yielding 10<sup>8</sup> cells (Nakajima *et al* 1984). The cell suspension was spun at 120 g for ten minutes at 4°C, and the cell pellet was then  
10 washed three times with 20 ml ice-cold PBS, pH 7.4. Washed cells were resuspended with 25 ml cold 50 mM Tris, pH 7.5, containing 1 mM phenylmethylsulphonylfluoride, 5 mM N-ethylmaleimide, 0.5% Triton X-100 and 0.05% sodium azide. After thirty minutes on ice, the extract was spun at 31000 g for thirty minutes at 4°C. The supernatant was concentrated using a Centriprep™-10 ultrafiltration device (Amicon  
15 Inc., USA) to a final volume of 0.6 ml. This concentrate is the 'enzyme solution' referred to in the enzyme assay below. Aliquots were snap-frozen and stored at -70°C.

An assay incubation mixture typically consists of the following ingredients, where heparin is sodium salt Grade 1A from Sigma and heparan sulphate is bovine kidney  
20 (super special grade) from Seikagaku.

	2.0 $\mu$ l	premix solution
	1.0 $\mu$ l	heparan sulphate (50 kDa) or heparin (20 kDa) in 20 mM NaP, pH 7.0
	1.0 $\mu$ l	inhibitor solution in water
	<u>1.0 <math>\mu</math>l</u>	enzyme solution
25	5.0 $\mu$ l	Incubate 37°C for ten hours

Six different incubation mixtures may then be loaded to a HD Phastgel™, (Pharmacia Corporation, Sweden - a 20% polyacrylamide gel containing 30% ethylene glycol) using  
30 an applicator designed to deposit 4 $\mu$ l per lane. The gel may then be electrophoresed

in a Pharmacia Phastsystem™ (Pharmacia Corporation, Sweden) using native buffer strips. Sample application is advantageously done using limit settings of 500V, 1 mA, 3W, while the separation is preferably done at 15°C using limit settings of 500V, 10 mA, 3W. The separation is best terminated when the electrophoresis ion front (visible as a thin brown band) has travelled about half-way through the resolving gel. The gel may then be stained for ten minutes using a 0.08% aqueous solution of Azure A (Sigma), and destained for approximately one hour under running tapwater. Heparin and heparan sulphate are stained preferentially by this procedure, and appear as purple bands or purple smears depending on the fragment size range. The effect of heparanase activity during the assay incubation is to convert the substrate from a high molecular weight glycosaminoglycan (seen as a dense low-mobility band) to degradation products of lower molecular weights (seen as a less dense smear between the substrate band position and the electrophoresis ion-front, or as an accumulation of stained material at the ion-front itself). The extent to which this process is prevented by an inhibitor in the assay mix may be assessed by comparing the inhibitor track to appropriate control tracks (for example, one from an assay lacking inhibitor, and another from an assay containing enzyme inactivated previously by boiling). Any protein in the assay that is able to migrate into the high density gel will be seen as a thin blue band if it is present in sufficient quantity, and BSA is an example of one such protein.

Radioassays for measuring heparitinase and heparanase activity in a quantitative manner were carried out as follows. Heparanase activities were measured by an adaptation of an existing method (Oldberg *et al* (1980) *Biochemistry* 19, 5755-5762) in which high M<sub>r</sub> [<sup>3</sup>H]heparin was used as substrate in place of [<sup>3</sup>H] heparan sulphate.

High M<sub>r</sub> [<sup>3</sup>H]heparin for use as a substrate in the enzyme assay was prepared by gel filtration, as follows. A 1.4 mg sample of Na[<sup>3</sup>H]heparin (Dupont NET 467, specific radioactivity 0.7 mCi/mg, prepared by sodium borotritide reduction of heparin) was applied to a column of Pharmacia Sephadex G50 Fine (27 x 0.9 cm, bed vol. 17.2 ml) equilibrated with 10 mM Na MES, pH 6, and chromatographed at 7.3 ml/h.

Radioactivity-containing fractions corresponding to the void volume were pooled to give 2.5 ml substrate solution ( $5.9 \times 10^4$  cpm/ $\mu$ l).

Enzyme assays (total volume of 25  $\mu$ l) were conducted in 10-50 mM Na MES, pH 6, containing platelet extract, 0.6 mg/ml BSA, 13  $\mu$ g/ml unlabelled heparin, and 187 000 cpm of high  $M_r$  [ $^3$ H]heparin. Assays contained a final concentration of  $\sim 2.1$   $\mu$ M heparin. After incubation at 37°C for sixteen and a half hours under mineral oil, two 5  $\mu$ l samples were taken from each reaction. Each sample was added to 395  $\mu$ l 28 mM Na MES, containing 12.7  $\mu$ g/ml heparin, 50 mM sodium acetate, and 0.3 M NaCl, pH 6; undegraded heparin was then precipitated by addition of 100  $\mu$ l 5% (w/v) cetyl pyridinium chloride. After incubation at 37°C for thirty minutes, the suspensions were centrifuged at 11300 g for thirty minutes room temperature. Any particulate material inadvertently collected with the supernatants was removed by centrifugation through a 0.45  $\mu$ m nylon filter (Lida, Series 8000). Samples (0.4 ml) of each supernatant were mixed with 5 ml Instagel scintillation fluid (Packard) and radioactivity measured by liquid scintillation (LKB Rackbeta). If the cpm values from the duplicate precipitations were not within 10% of their mean, the assay was repeated.

## EXAMPLE 2

### Synthesis, purification and use of phosphorothioate oligonucleotides

The synthesis and purification of phosphorothioate oligonucleotides is a routine process and can be carried out according to Zon and Geiser (1991) *Anticancer Drug Des.* 6, 539-568. Syntheses of phosphodiester and phosphorothioate oligonucleotides were carried out using an automatic DNA synthesizer (PCR-Mate, Model 391, from Applied Biosystems, USA) according to the manufacturers instructions. The sulphurization reagent was tetraethylthiuram disulphate (Applied Biosystems). Each oligonucleotide was cleaved from its support using concentrated ammonia, and the cleaved oligonucleotide was deprotected by incubating the ammoniacal solution for sixteen hours at 55°C. Each deprotected oligonucleotide was then purified by chromatography using a

NAP-10 gel filtration column (Pharmacia), or an Oligonucleotide Purification Cartridge (OPC™) from Applied Biosystems, according to the manufacturers instructions.

The synthesized oligonucleotides are shown in Table 1.

TABLE 1

Details of oligonucleotides synthesized

All oligonucleotides were single stranded and were chemically synthesized and purified as described in this example. In the table, A denotes a dA nucleotide residue; C a dC nucleotide residue; G a dG nucleotide residue; and T a dT nucleotide residue.

Code	DNA type	Length (nucleotides)	Sequence
LG1PO	Phosphodiester	15	5'-GGTTGGTGTGGTGG-3' (Seq ID No 1)
LG1PS	Phosphorothioate	15	5'-GGTTGGTGTGGTGG-3' (Seq ID No 3)
LG2PS	Phosphorothioate	7	5'-GGGTTGG-3' (Seq ID No 2)
LG3PS	Phosphorothioate	15	5'-AAACCAACCACAAAC-3' (Seq ID No 5)
LG4PS	Phosphorothioate	30	5'-GGGTTGGTTGTGGGTTGGGTTGGTTGTGGGTT-3' (Seq ID No 6)
LG5PS	Phosphorothioate	30	5'-GGGTCGGGTCGGGTCGGGTCGGGTCGGGTC-5' (Seq ID No 10)
LG6PS	Phosphorothioate	15	5'-GGGTTGGTTGTGGGTT-3' (Seq ID No 4)
SIM-AS	Phosphorothioate	18	5'-GTGTCGGGTCCTCCGGGC-3' (Seq ID No 7)
SIM-S	Phosphorothioate	18	5'-GCCCCGAGACCCGACAC-3' (Seq ID No 8)
SIM-CON	Phosphorothioate	18	5'-GTGCCGGGTCCTCCGGGC-3' (Seq ID No 9)

Phosphorothioate oligonucleotides purified by OPC™ columns (Applied Biosystems) or NAP-10 columns (Pharmacia), were shown to be free from extraneous heparanase-inhibiting contaminants as follows. A sample of LG1PS (Table 1) was synthesized without retention of the trityl group and cleaved from the column as described above. The oligonucleotide was then purified by preparative gel electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Oligonucleotide was extracted from the gel slice and concentrated by extraction with n-butanol and ethanol precipitated; a gel slice

of equal size, but lacking DNA, was used as a control sample. Extracts were then purified further by gel filtration on Pharmacia Nucleic Acid Purification™ (NAP-10) (Pharmacia Corporation, Sweden) columns according to the manufacturers instructions. Additional purification by ion-exchange chromatography was done using Pharmacia  
5 DEAE-Sepharose Fast Flow™ (Pharmacia Corporation, Sweden). A solution of 15 mM ammonium acetate, pH 6.0, was used to equilibrate and wash the columns (2 ml bed volume). Columns were developed by increasing the concentration of ammonium acetate to 7.5 M, and by washing with 2.5 M ammonium acetate, pH 9.4. Bound LG1PS was then eluted using a solution of 2.5 M sodium chloride that had been adjusted to pH 2  
10 using glacial acetic acid. The eluted oligonucleotide was ethanol precipitated. Oligonucleotide LG1PS purified in this way inhibited human platelet heparanase to the same extent as LG1PS that had been purified using a single OPC™ column, or a single NAP-10 column (see above). When the extract from the control polyacrylamide gel slice was subjected to gel-filtration, ion-exchange and ethanol precipitation steps (all as  
15 described) the resulting sample did not inhibit the enzyme.

Tables 2 through 4 set out the results of inhibition of bacterial heparinases and mammalian heparinases by phosphorothioate oligonucleotides. As shown in these tables phosphorothioate oligonucleotides are potent inhibitors of glycosaminoglycan degrading  
20 enzymes. It is evident from Tables 2 through 4 that the relative potencies of different phosphorothioate oligonucleotides as inhibitors of bacterial heparinases are reflected in their relative potencies with mammalian heparanase.

TABLE 2  
Inhibition of bacterial glycosaminoglycan-degrading enzymes by phosphorothioate oligonucleotides

Oligo-nucleotide		Comp	Heparinase I					Heparinase II	Chondroitinase	
Substrates ( $\mu$ M):										
Chondroitin sulphate									58 58	
Heparin			38	38	38	38	38	38		
Heparin (20 kDa)										
Heparan sulphate (50 kDa)										
Inhibitors ( $\mu$ M):										
LG1PD	PD	15	8 & T	5				5		
LG2PS	PS	7	G & T	5						
LG1PS	PS	15	G & T		5	18		16		
LG6PS	PS	18	G & T		5		16			
LG3PS	PS	15	A & C		5					
LG4PS	PS	30	G & T			5			5 0.05	
%Inhibition			0	9	78	82	27	99	85 94	0 89 73 0

15

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**TABLE 4**  
**Inhibition of platelet heparanase by phosphorothioate oligonucleotides**

Substrates ( $\mu$ M):	Oligo-nucleotide	Comp	Platelet heparinase
Heparin (20 kDa)			
Heparan sulphate (50 kDa)			
Inhibitors ( $\mu$ M):			
LG1P0	P0	15	G&T
LG2PS	PS	7	G&T
LG1PS	PS	15	G&T
SIM-AS	PS	18	G,T&C
LG8PS	PS	15	G&T
SIM-S	PS	18	G,C&A
LG3PS	PS	15	G&T
SIM-CON	PS	18	G,T&C
LG4PS	PS	30	G&T
Inhibition			



With reference to Tables 2 through 4:

Platelet heparanase is from human platelets, melanoma heparanase from melanoma cells, bacterial heparinases are from *Flavobacterium heparinum* and chondroitinase is from *Proteus vulgaris*.

5 Oligonucleotides are identified by the codes defined in Table 1

The abbreviations used are as follows:

	nt	oligonucleotide length in nucleotide base units
	Comp	composition (where A denotes a dA nucleotide residue, C a dC
10		nucleotide residue, G a dG nucleotide residue, and T a dT
		nucleotide residue);
	kDa	kilo Daltons. Nominal chain sizes are shown for size-
		fractionated substrates.
	Inhibition	Scores are derived from polyacrylamide microgel assays as
15		described in Example 1 and mean:
	-	no inhibition
	+	slight inhibition
	++	partial inhibition
20	+++	full inhibition.

In a second experiment LG1PS and LG1PO were tested for inhibition of platelet heparanase (by radioassay) together with suramin, D-glucuronic acid, D-glucosamine-2-sulphate, D-saccharic acid-1,4-lactone, D-glucuronic acid-1,5-lactone, deoxy-  
 25 nojirimycin, D-glucuronic acid-1,5-lactam, and  $\beta$ -D<sup>4</sup>GlcA(S)-(1-4)-GlcN(S)-disaccharides. Results are shown in Table 5.

TABLE 5

Compounds tested for inhibition of platelet heparanase

COMPOUNDS	INHIBITION (%) <sup>a</sup>	Concentration (μM)
Suramin	50	350 <sup>b</sup>
LG1PS (15-mer)	50	470 <sup>b</sup>
LG1PD (15-mer)	0	500
D-glucuronic acid	0	500
D-glucosamine-2-sulphate	0	500
D-saccharic acid-1,4-lactone	0	22000
D-gluconic acid-1,5-lactone	0	500
Deoxyribojirimycin	0	500
D-gluconic acid-1,5-lactam	0	500
β-Δ <sup>4</sup> GlcA(S)-(1,4)-GlcN(S) disaccharides	0	3000

<sup>a</sup> Determined by heparanase radioassay; <sup>b</sup>IC<sub>50</sub> values obtained by titration

The effect on inhibition of phosphorothioate oligonucleotide length and composition on inhibition of platelet heparanase is shown in Table 6.

TABLE 6

Effect of PS oligonucleotide length and  
composition on inhibition of platelet heparanase

PS OLIGONUCLEOTIDE	COMPOSITION	LENGTH	INHIBITION (%) <sup>a</sup>	CONCENTRATION (μM)
LG2PS	GT	7	0	500
LG1PS	GT	15	50	470 <sup>b</sup>
LG3PS	AC	15	0	500
LG4PS	GT	30	50	28 <sup>b</sup>

<sup>a</sup> Determined by heparanase radioassay; <sup>b</sup>IC<sub>50</sub> values obtained by titration

As shown in Table 5, only phosphorothioate oligonucleotides and suramin were observed to cause significant inhibition of platelet heparanase.

As is evident from Tables 2 through 6, phosphorothioate oligonucleotides, consisting of dG and dT nucleotides inhibit platelet heparanase better than those consisting of dA and dC nucleotides. The inhibitory effect of phosphorothioate oligonucleotides also increases with length (Table 6).

One of the more potent oligonucleotides tested is LG4PS, a 30-mer phosphorothioate oligonucleotide consisting of dG and dT nucleotides. The sequence of this oligonucleotide is given in Table 1. This oligonucleotide causes significant inhibition (approximately 30%) of platelet heparanase at 15  $\mu$ M (with heparin as substrate) and at 3  $\mu$ M (with heparan sulphate as substrate).

The fact that the phosphorothioate but not the phosphodiester version of the LG1 sequence caused inhibition (Table 1) suggested that the ability of oligonucleotides to inhibit heparanase was confined to sulphur-substituted species. Inhibition by a highly purified preparation of LG1PS confirmed that the effect was directly due to the oligonucleotide.

Since inhibitory potency was affected by base composition, experiments were conducted to see if additional potency (and perhaps specificity) could be conferred by manipulating the nucleotide sequence. A positional scanning procedure suitable for use with oligonucleotide analogues (Ecker *et al* (1993) *Nucleic Acids Res.* 22, 3202-3209) was employed to optimize the inhibition of platelet heparanase. This entails repeatedly synthesizing and testing a set of four phosphorothioate oligonucleotide pools. In the positional scanning of a PS DNA 11-mer (Table 7), G was clearly preferred up to and including round 4, but not thereafter. In round 5, G and C gave comparable results, while in rounds 6 and 7 all four nucleotides gave comparable results. A direct comparison of the winning oligonucleotide pools from rounds 1 to 7 is shown in Table

7. As no real gains in efficacy had been made since round 5, the scanning was discontinued. The outcome of the scanning experiments has precedent in that several studies of PS-oligonucleotides have implicated G<sub>3</sub> or G<sub>4</sub> motifs as the pharmacophores responsible for non-antisense effects such as antiviral activity (Ecker *et al* (1993) *Nucleic Acids Res.* 22, 3202-3209 and Rando *et al* (1995) *J. Biol. Chem.* 270, 1754-1760).

When a PS DNA 30-mer containing five G<sub>4</sub> repeats (LG5PS, consisting of tandem repeats of the preferred motif GGGGTC) was tested as an inhibitor of platelet heparintinase in vitro it had an IC<sub>50</sub> of 24  $\mu$ M, almost the same as that of the original 30-mer (LG4PS) which contained four G<sub>3</sub> repeats. The increase in length from eleven nucleotides (Table 7, round 7) to thirty nucleotides (LG5PS) was accompanied by a 3.8-fold decrease in IC<sub>50</sub> value. With hindsight, it can be assumed that much of the 18-fold difference in IC<sub>50</sub> value between 15-mer LG1PS and 30-mer LG4PS (Table 6) was in fact due to the presence of G<sub>3</sub> motifs in LG4PS but not LG1PS. Overall, it was concluded that the inhibitory efficacy of a PS oligonucleotide was greatly enhanced by the presence of a single internal GGG motif, but that further improvements depended more on substantial increases in length than on the composition or sequence of the remainder of the oligonucleotide. Despite expectations based on other work (Fujihashi *et al* (1994) *Biochem. Biophys. Res. Commun.* 203, 1244-1250), it was found that 100  $\mu$ M of the DNA or RNA forms of the PS 4-mer 5'-GGGG-3' did not inhibit platelet heparanase, even when they were 5'-phosphorylated.

The inhibitory effects of PS oligonucleotides were not limited to platelet heparanase. In the PhastGel assay, the heparan sulphate-degrading activities of both platelet and murine melanoma cell lysates were fully inhibited by 310  $\mu$ M LG1PS. With both of these enzymes, it was observed that HS degradation was much more sensitive to inhibition than heparin degradation. Estimates of inhibitor efficacy from PhastGel assays that employed heparin as substrate agreed well with IC<sub>50</sub> values determined by the radioassays. Estimates from PhastGel assays that utilized HS as substrate suggested

that 3  $\mu$ M LG4PS caused ~30% inhibition of platelet heparintinase. In a quantitative radioassay, the hydrolysis of HS by a human tumour heparanase was inhibited by LG4PS with an  $IC_{50}$  value of 1.4  $\mu$ M. PhastGel assays (see Example 1) done using mouse macrophage heparanase at pH 6 (in the absence of sodium chloride) with ~26  $\mu$ M heparin as substrate suggested that LG4PS had an  $IC_{50}$  value of about 28  $\mu$ M. Similar assays done with ~23  $\mu$ M HS as substrate suggested an  $IC_{50}$  of about 15  $\mu$ M for LG4PS.

TABLE 7

Optimization of PS inhibition by positional scanning

Round	5'-Sequence-3'	Preferred X	Inhibition (%) <sup>a</sup>
1	NNNNNXNNNNN	G	1
2	NNNNXGNNNN	G	15
3	NNNNGGNNNN	G	78
4	NNNXGGGNNNN	G	76
5	NNNGGGGXNN	C	74
6	NXNGGGGNCNN	T	73
7	NTNGGGGXCNN	T	82

<sup>a</sup> Determined by heparanase radioassay at total oligonucleotide concentration of 125  $\mu$ M

It was found that the inhibitory effect extended to bacterial GAG-degrading enzymes. Heparinase I was 80% inhibited by 5  $\mu$ M LG1PS, but was unaffected by 5  $\mu$ M LG1PO. Once again, inhibitory efficacy increased with oligonucleotide length and guanosine content (Table 2). Time courses (that is, plots of  $A_{232}$  against time) of PS-inhibited heparinase I reactions were curved during the first few minutes, unlike their uninhibited counterparts; this was interpreted to mean that PS-enzyme binding was slow. Heparinase II and chondroitinase ABC were inhibited by PS oligonucleotides in a manner similar to heparinase I (Table 2).

The significance of oligonucleotide-G motifs appears to derive from the ability of contiguous guanosine to assemble into stacks of G-quartets, either through intramolecular (Rando *et al* (1995) *J. Biol. Chem.* 270, 1754-1760 and Schultze *et al* (1994) *J. Mol. Biol.* 235, 1532-1547) or intermolecular (Wyatt *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91, 1356-1360; Bennett *et al* (1994) *Nucleic Acids Res.* 22, 3202-3209 and Laughlan *et al* (1994) *Science* 265, 520-524), interactions. The stable framework which results forms a basis for aptameric interactions with proteins (Wang *et al* (1993) *Biochemistry* 32, 11285-11292 and Padmanabhan *et al* (1993) *J. Biol. Chem.* 268, 17651-17654). Since intermolecular quartets may be disrupted temporarily by heating, with consequent loss of aptamer activity (Bennett *et al* (1994) *Nucleic Acids Res.* 22, 3202-3209), we tested the effect of heating a LG4PS solution before adding it to the rapid heparinase I assay. Incubation at 100°C for ten minutes had no effect on inhibition, suggesting that in this case oligomerization was not a prerequisite for inhibition.

### EXAMPLE 3

#### Oligonucleotides containing varying numbers of sulphur-substituted linkages

A series of 15-mer oligonucleotides comprising from 0 to 14 sulphur substituted backbone linkages are synthesized according to the procedure of Example 2. The oligonucleotide has the following base composition:

5' GGTGTTGGTGTGGTTGG 3'

Phosphorothioate linkages are successively incorporated from the 5' end through to the 3' end. Resultant oligonucleotides are assayed for glycosaminoglycan-degrading enzymes inhibitory activity. Results are set out in Table 8 below where a "•" indicates a phosphorothioate linkage.

TABLE 8

	Compound	Platelet heparanase inhibition
	GGTTGGTGTGGTTGG	-
5	G•GTTGGTGTGGTTGG	+
	G•G•TTGGTGTGGTTGG	+
	G•G•T•TGGTGTGGTTGG	+
	G•G•T•T•G•GTTGGTTGG	+
	G•G•T•T•G•G•GTTGGTTGG	+
	G•G•T•T•G•G•G•GTTGGTTGG	++
10	G•G•T•T•G•G•G•G•GTTGGTTGG	++
	G•G•T•T•G•G•G•G•G•GTTGGTTGG	++
	G•G•T•T•G•G•G•G•G•G•GTTGGTTGG	++
	G•G•T•T•G•G•G•G•G•G•G•GTTGGTTGG	++
	G•G•T•T•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
15	G•G•T•T•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
20	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•G•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++
	G•G•T•T•GTTGGTTGG	+++

20

- no inhibition;      + slight inhibition  
 ++ partial inhibition      +++ complete inhibition

25

This example shows that increasing the proportion of phosphorothioate linkages within the oligonucleotide increases inhibitory effects on mammalian heparanases. Having said this, oligonucleotides containing a mixture of phosphorothioate and phosphodiester linkages also show inhibitory activity towards heparanases.

## EXAMPLE 4

Phosphorothioate oligonucleotides as agents for preventing  
the activation and proliferation of rabbit vascular smooth muscle cells

- As detailed explained, one aspect of the invention provides PS oligonucleotides which are useful for suppressing the activation, migration and proliferation of vascular smooth muscle cells (SMC) that occurs during vascular hyperplasia. During hyperplasia, vascular SMC change from their quiescent form (known as the 'contractile phenotype') into the noncontractile form associated with mitogen-responsiveness, migration, proliferation and synthesis of extracellular matrix (known as the 'synthetic phenotype').
- Cells exhibiting the latter phenotype are considered to be activated, and are definitively characterised by a low volume density of myofilament in the cytoplasm (Campbell & Campbell, (1993) *Clinical Science* 85, 501-513; Campbell *et al*, (1992) *Exp. Cell Res.* 200, 156-167).
- Primary (that is, contractile) rabbit aortic SMC were harvested from the aortic media of nine to twelve week old rabbits by enzyme dispersion (Campbell & Campbell, (1993) *Clinical Science* 85, 501-513) and seeded in 96-well or 24-well tissue culture plates on day 0. The SMC were cultured in growth medium consisting of M199 and Hank's salts, supplemented with 5% foetal calf serum, 2 mM glutamine, and penicillin (0.6 mg/ml). On day one the growth medium was removed and replaced with fresh medium containing LG4PS at the concentrations shown in Table 9. Treatments were in triplicate (that is,  $n = 3$ ) and the medium was not changed thereafter. On day three the cells were pulsed with [ $^3\text{H}$ ]thymidine at 1  $\mu\text{Ci/ml}$  in growth medium for five hours and harvested onto glass fibre filters. The radioactivity collected on the filters was then determined by liquid scintillation counting. Table 8 shows the results of four different experiments, each of which is shown on a different row of the Table.



TABLE 9

Effects on activation and growth of rabbit primary SMC

LG4PS $\mu$ M	0	0.05	0.3	1	30
Seeded $3.3 \times 10^4$ cells/cm <sup>2</sup>	$26 \pm 3$	nd	nd	$11 \pm 1^*$	$0.8 \pm 0.1^*$
Seeded $1.3 \times 10^5$ cells/cm <sup>2</sup>	$109 \pm 7$	$85 \pm 8^*$	$80 \pm 9^*$	$54 \pm 3^*$	nd
Seeded $1.3 \times 10^5$ cells/cm <sup>2</sup>	$206 \pm 13$	$221 \pm 9$	$184 \pm 9$	$149 \pm 9^*$	nd
Seeded $1.1 \times 10^5$ cells/cm <sup>2</sup>	$436 \pm 37$	$335 \pm 26$	$308 \pm 26^*$	$218 \pm 37^*$	nd

- 10 The values shown in Table 9 are  $10^{-2} \times$  (dpm incorporated  $\pm$  sem), and are indicative of cell proliferation, an asterisk (\*) denotes a statistically significant difference from the appropriate no-PS value ( $p < 0.05$ ). The data indicate that the activation and/or proliferation of rabbit primary smooth muscle cells may be inhibited by concentrations of LG4PS as low as 0.3  $\mu$ M, and show that they are certainly inhibited by 1  $\mu$ M
- 15 LG4PS. The extent of inhibition at the latter concentration is in the range 28% to 58%, and is typically around 50%.

Table 10 shows the results of an experiment done with rabbit aortic SMC that were already in the synthetic phenotype before treatment with LG4PS. Rabbit SMC that had

20 been subcultured four times were seeded on day 0 in 24-well tissue-culture trays at  $5 \times 10^4$  cells/well in growth medium. On day one, triplicate wells received L4PS at the final concentrations shown in the Table; the no-PS controls received growth medium alone. On day three the cells were pulsed with [<sup>3</sup>H]thymidine at 1  $\mu$ Ci/ml in growth medium for five hours and harvested onto glass fibre filters. The radioactivity collected

25 on the filters was then determined by liquid scintillation counting.

TABLE 10

Effects on growth of proliferating rabbit SMC

LG4PS $\mu$ M	0	0.05	0.3	1	3	10
Growth	20.7 $\pm$ 0.4	20.8 $\pm$ 0.2	6 $\pm$ 1*	4.0 $\pm$ 0.3*	2.8 $\pm$ 0.4*	1.8 $\pm$ 0.2*
Viability %	89	87	88	88	80	74

The values shown in the top row of Table 10 are  $10^{-2} \times$  (dpm incorporated  $\pm$  sem), and an asterisk (\*) denotes a statistically significant difference in [ $^3$ H]thymidine uptake from the appropriate no-PS control value ( $p < 0.05$ ). Cytotoxic effects of LG4PS were estimated by using Trypan Blue dye exclusion to assess cell viability on day three; the results are shown in the bottom row of the Table. Overall, the data indicate that anti-proliferative effects are observed for LG4PS at concentrations of 0.3  $\mu$ M and above, whereas cytotoxic effects are not observed until concentrations of 3  $\mu$ M to 10  $\mu$ M are used.

As mentioned herein macrophages stimulate the activation of SMC by way of their heparanase activity (Campbell *et al.*, (1992) *Exp. Cell Res.* 200, 156-167). An experiment was done to test whether the macrophage-stimulated activation (and/or the subsequent proliferation) of SMC was affected by PS oligonucleotides. On day 0, primary (that is, contractile) rabbit aortic SMC were seeded in growth medium at  $4 \times 10^4$  cells/well in 96-well tissue-culture plates, either with or without macrophages ( $1.3 \times 10^4$  cells/well). On day one, triplicate wells received L4PS at the final concentrations shown in Table 11; the no-PS controls received growth medium alone. The medium was not changed thereafter. On day three the cells were pulsed with [ $^3$ H]thymidine at 1  $\mu$ Ci/ml in growth medium for five hours and harvested onto glass fibre filters. The radioactivity collected on the filters was then determined by liquid scintillation counting.

TABLE 11

Effects on activation and growth of macrophage-stimulated primary rabbit SMC

LG4PS $\mu$ M	0	0.05	0.3	1
Macrophage only	2.1 $\pm$ 0.4	5 $\pm$ 1	3 $\pm$ 2	5 $\pm$ 3
SMC only	206 $\pm$ 13	221 $\pm$ 9	184 $\pm$ 9	149 $\pm$ 9*
SMC + Macrophage	301 $\pm$ 26*	305 $\pm$ 5*	223 $\pm$ 15	184 $\pm$ 85

5 The values shown in Table 11 are  $10^{-2} \times$  (dpm incorporated  $\pm$  sem) for this experiment, and an asterisk (\*) denotes a statistically significant difference from the appropriate no-PS SMC-only control value ( $p < 0.05$ ). The other symbol (\$) denotes difference from the no-PS SMC+macrophage control value ( $p < 0.05$ ). As expected, macrophages did stimulate the activation and/or proliferation of contractile SMC, but this stimulation was prevented by the presence of LG4PS at 0.3  $\mu$ M or above. (At 0.3  $\mu$ M, LG4PS had no effect on unstimulated SMC.) As explained above, some or all of the prevention of stimulation observed here is likely to result from the inhibition of macrophage heparanase by LG4PS.

20 To demonstrate the effects of LG4PS on SMC activation in isolation from any effects on the proliferation of activated SMC, the following experiment was done. On day 0, primary (that is, contractile) rabbit aortic SMC were seeded in growth medium at  $1.1 \times 10^6$  cells/well in a 6-well tissue-culture plate, either with or without macrophages ( $3.1 \times 10^5$  cells/well). On day one, the medium was replaced with fresh medium containing LG4PS at the concentrations specified in Table 12. On day three the cells were fixed in glutaraldehyde and were processed for transmission electron microscopy. At least twenty photomicrographs from each of two to three replicates per treatment were analyzed morphometrically by point counting (Mosse *et al.*, (1985) *Lab. Invest.* 53, 555-562). The volume fraction of myofilament (% V<sub>v</sub> myo) was determined as 100 x volume fraction of myofilament / volume fraction of cytoplasm.

30

TABLE 12

Effects on activation of macrophage-stimulated primary rabbit SMC

LG4PS $\mu$ M	0	0.3	1	3	10
SMC only	44.9 $\pm$ 0.4	43.4 $\pm$ 0.8	nd	nd	nd
SMC + macrophage	37.9 $\pm$ 1.3*	45.5 $\pm$ 0.85	42.1 $\pm$ 1.55	43.4 $\pm$ 1.75	50.0 $\pm$ 2.25*

The values obtained for  $V_{\text{myo}}$  are shown in Table 12; lower values are indicative of SMC activation. An asterisk (\*) denotes a statistically significant difference from the appropriate no-PS SMC-only value ( $p < 0.05$ ). The other symbol (\$) denotes difference from the no-PS SMC+macrophage control ( $p < 0.05$ ). The  $V_{\text{myo}}$  of contractile SMC was significantly reduced by co-culture with macrophages, thereby confirming the macrophage-mediated promotion of SMC activation. In agreement with the results obtained in Table 10, the presence of LG4PS at 0.3  $\mu$ M or above prevented the macrophage-mediated stimulation of SMC phenotype change. (At 0.3  $\mu$ M, LG4PS had no effect on the  $V_{\text{myo}}$  value of unstimulated SMC.) As explained above, some or all of the prevention of stimulation observed here is likely to result from the inhibition of macrophage heparanase by LG4PS.

## EXAMPLE 5

Phosphorothioate oligonucleotides as agents for preventingthe activation and proliferation of human and pig vascular smooth muscle cells

The effect of LG4PS on the proliferation of human vascular SMC was investigated as follows. Small pieces of mammary artery, redundant from coronary by-pass operations, were used to set up explant cultures of the media as described (Neylon *et al.*, (1990) *Circulation Res.* 67: 814-825). The culture medium was M199 with Earles salts containing 20% foetal calf serum, antibiotics (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin sulphate) and extra glutamine (to 6 mM). Cells were used at passage 2

to 6 from the primary explant and were in the synthetic phenotype. Cells were seeded in 96-well tissue culture plates at  $1.3 \times 10^3$  cells per well in 100 $\mu$ l complete culture medium containing 15% foetal calf serum on day 0. On day one replicates of 6 wells received a further 100 $\mu$ l of the same culture medium containing each inhibitor to give the final concentrations shown in Table 13. On day one, before addition of inhibitors, some wells were treated with MTS (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions, to estimate cell number from the absorbance of the coloured formazan produced. On day three the cultures were fed by replacing half the medium with fresh medium containing appropriate inhibitors. On day seven or eight a second MTS assay was done and the increase in cell number was estimated. Percentage inhibition of proliferation was calculated from the formula:

$$((C-T) / C) \times 100$$

where C = increase in MTS absorbance of untreated control, T = increase in MTS absorbance of inhibitor treatment. Isolate numbers in Table 13 refer to two different sources of human SMC.

TABLE 13  
Effect of LG4PS on proliferation of human VSMC

----- % Inhibition -----			
Inhibitor	Concentration $\mu$ M	Isolate 1	Isolate 2
LG4PS	10	34.4*	47.5*
LG4PS	1	5.0	1.23
Heparin	100 $\mu$ g/ml (5 to 10 $\mu$ M)	13.1*	38.0*

An asterisk (\*) denotes statistically significant inhibition ( $p < 0.05$ , Analysis of Variance and Student Neuman Keuls test). LG4PS at 10  $\mu$ M concentration showed a degree of inhibition of proliferation of human VSMC that compared favourably with a

similar concentration of heparin (a well known inhibitor of SMC proliferation), whereas LG4PS at 1  $\mu$ M was without effect.

The effect of LG4PS on the proliferation of pig aorta SMC was investigated using passage 1 cells from explants used in the control wells of the experiment described below. The cells were in the synthetic phenotype. The proliferation test was done as described for the human cells except that the serum concentration was 10%, cell seeding concentration was  $3 \times 10^2$  or  $6 \times 10^2$  per well and the assay was completed on day four or five without further feeding. These differences were due to the much more rapid proliferation rate of the pig cells compared with the human cells. The results are shown in Table 14.

TABLE 14  
Effect of LG4PS on proliferation of pig VSMC

----- % Inhibition -----			
Inhibitor	Concentration $\mu$ M	300 cells/well	600 cells/well
LG4PS	5	78* .2	68.8*
LG4PS	1	34.5*	20.0
LG4PS	0.3	20.2*	26.8

An asterisk (\*) denotes statistically significant inhibition ( $p < 0.05$ , Analysis of Variance & Student Newman Keul's test). Cells seeded at the lower concentration were inhibited significantly by all three concentrations of LG4PS, whereas at the higher cell seeding concentration only 5  $\mu$ M LG4PS caused significant inhibition.

LG4PS was also tested for its effect in an in vitro model of SMC migration from the medial layers of damaged arteries. Fresh pig aortas were obtained from a local abattoir and maintained overnight at 4°C in Hank's balanced salt solution containing double-strength antibiotics. Connective tissue was removed and the aorta cut into pieces 2.5 cm long. They were processed as for the human SMC explant cultures but at the

stage of peeling small sections of media from the adventitia, larger pieces were pulled off. Uniform round pieces of media were obtained using a 2 mm diameter biopsy punch (Stiefel Laboratories), each being placed in the centre of a well of 96-well tissue culture plate in a minimum volume (20  $\mu$ L to 30  $\mu$ L of culture medium as for human SMC but containing 10% foetal calf serum). The wells of the culture plate had been previously coated with gelatin by a two hour incubation with 100  $\mu$ L per well sterile 1% gelatin (Sigma) in phosphate buffered saline for two hours at 37°C. The gelatin solution was aspirated before addition of tissue. Following overnight incubation at 37°C (that is, day one) the volume of each well was made up to 50  $\mu$ L with complete medium and the plates were reincubated for four hours. Then a further 50  $\mu$ L of culture medium containing inhibitors was added per well, to give the final concentrations shown in Table 15, 30-36 wells being used for each treatment. At day four each well received an additional 100  $\mu$ L of appropriate culture medium. On days four through seven the wells were examined microscopically for the appearance of SMC separate from the explanted tissue. Those wells having  $\geq 10$  such cells visible were scored as positive. This assay measures the combined effects of SMC activation and migration and may also include a component of proliferation, although by taking the endpoint as the appearance of a small number of migrated cells, the contribution of proliferation should be minimal. Effects of treatments were analyzed when the number of positives in the inhibitor-free treatments fell between 10 and 25. The statistical significance of particular treatments was analyzed by a Binomial significance test. Table 15 shows results of two experiments. Percent inhibition was calculated from the formula:

$$((P_c - P_t) / P_c) \times 100$$

where  $P_c$  = number of positives in control treatment,  $P_t$  = number of positives in inhibitor treatment.

TABLE 15

Effect of LG4PS on pig media explant outgrowth

----- % Inhibition -----			
Inhibitor	Concentration $\mu$ M	Experiment 1	Experiment 2
LG4PS	10	100.0*	95.0*
LG4PS	5	81.5*	79.2*
LG4PS	1	nd	10.0
Heparin	100 $\mu$ ml (5 to 10 $\mu$ M)	85.7*	78.9*

An asterisk (\*) denotes statistically significant inhibition ( $p < 0.05$ ). LG4PS at 10  $\mu$ M concentration was a very effective inhibitor of SMC migration from the explant and at 5  $\mu$ M compared favourably with heparin, whereas at 1  $\mu$ M it was without effect.

The cytotoxicity of LG4PS for human and pig SMC was estimated using the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl] tetrazolium bromide) to coloured formazan product (Promega), according to the manufacturer's instructions. MTT is used to measure cell numbers in cell culture and the cytotoxicity of agents applied to cultured cells (Carmichael *et al*, (1987) *Cancer Res.* 47, 936). SMC of either type were seeded into wells of a 96-well plate and grown to confluence in culture medium containing serum at 20% for human cells and 10% for pig cells. Six replicates of each cell type were stained with crystal violet as described (Kueng *et al* (1989) *Anal. Biochem.* 182:16) to give an estimate of cell number from the crystal violet absorbance. Other sets of twelve replicates received inhibitors at the concentrations shown in Table 15 (or no inhibitor) and were incubated at 37°C overnight. Six replicates of each treatment were then stained with crystal violet and the other six were assayed for MTT conversion. The crystal violet absorbances were compared across the treatments by Analysis of Variance. There was no significant difference between treatments and controls for either of the human SMC isolates ( $p > 0.05$ , Analysis of Variance) indicating no difference in cell number and suggesting that the cells had not proliferated



significantly in the twenty-four hour incubation. The conversion of MTT could therefore be compared directly between treatments. Table 16 shows that there was significant cytotoxicity for the human cells with increasing concentration of LG4PS.

TABLE 16

Cytotoxicity of LG4PS

Inhibitor	$\mu\text{M}$	—— % Inhibition of conversion of MTT ——		
		hu VSMC 235	hu VSMC 239	pig VSMC
LG4PS	1	0.0	0.0	1.9
LG4PS	5	12.9*	6.3	-1.7
LG4PS	10	14.5*	15.6*	-0.4
LG4PS	20	19.7*	18.7*	0.0
Heparin	100 $\mu\text{g/ml}$ (5 to 50 $\mu\text{M}$ )	13.3*	9.5	5.6

An asterisk (\*) denotes  $p < 0.05$ , Analysis of Variance & Student Newman Keul's test. At the biologically relevant concentrations of 5  $\mu\text{M}$  to 10  $\mu\text{M}$ , however, the cytotoxicity was low, and comparable to that of heparin, which is not considered to be very cytotoxic. With the pig cells there was a statistically significant difference between crystal violet absorbances for the different treatments ( $p < 0.05$ , Analysis of Variance), indicating that the cells had proliferated in the twenty four period and inhibitory effects of LG4PS on proliferation were detectable. The conversion of MTT was therefore corrected for the crystal violet absorbance before testing for statistically significant differences. Table 16 shows that none of the treatments caused detectable cytotoxicity for pig SMC's over and above effects on proliferation.

It is understood that scientific and other publications referred to throughout this specification are incorporated by reference in their entirety.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Cardiac Nominees Pty Ltd
- (ii) TITLE OF INVENTION: Glycosaminoglyan-degrading enzyme inhibition and resultant disease therapies
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: DAVIES COLLISON CAVE
- (B) STREET: LEVEL 10, 10 BARRACK STREET
- (C) CITY: SYDNEY
- (D) STATE: NEW SOUTH WALES
- (E) COUNTRY: AUSTRALIA
- (F) ZIP/POSTCODE: 2000
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: STEARNE DR, PETER A
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- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: +612 262 2611
- (B) FACSIMILE: +612 262 1080

## (2) INFORMATION FOR SEQ ID No 1:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	15 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 1

GGTTGGTGTGGTTGG

15

## (2) INFORMATION FOR SEQ ID No 2:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	7 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 2

GGGTTGG

7

## (2) INFORMATION FOR SEQ ID No 3:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	15 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii)	MOLECULE TYPE:	Oligonucleotide
------	----------------	-----------------

(xi)	SEQUENCE DESCRIPTION:	SEQ ID No 3
------	-----------------------	-------------

GGTTGGTGTGGTTGG

15

## (2) INFORMATION FOR SEQ ID No 4:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	15 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii)	MOLECULE TYPE:	Oligonucleotide
------	----------------	-----------------

(xi)	SEQUENCE DESCRIPTION:	SEQ ID No 4
------	-----------------------	-------------

GGGTTGGTTGTGGGT

15

## (2) INFORMATION FOR SEQ ID No 5:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	15 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 5

AAACCAACCACAAAC

15

## (2) INFORMATION FOR SEQ ID No 6:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	30 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 6

GGGTTGGTTGTGGGTGGGTTGGTTGTGGGT

30

## (2) INFORMATION FOR SEQ ID No 7:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	18 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 7

GTGTCGGGGTCTCCGGGC

18

## (2) INFORMATION FOR SEQ ID No 8:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	18 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 8

GCCCCGAGACCCCGACAC

18

## (2) INFORMATION FOR SEQ ID No 9:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	18 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID No 9

GTGCCGGGGTCTTCGGGC

18

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	30 bases
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGTCGGGG TCGGGGTCGG GGTCGGGGTC

30



The claims defining the invention are as follows:

1. A method for the inhibition of a glycosaminoglycan-degrading enzyme which comprises reacting said enzyme or cells producing said enzyme with an inhibitory effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms.
2. A method according to claim 1, wherein said oligonucleotide wholly or partially contains phosphorothioate linkages between adjacent nucleosides.
3. A method according to claim 1, wherein said oligonucleotide wholly or partially contains phosphorodithioate linkages between adjacent nucleosides.
4. A method according to any one of claims 1 to 3, wherein said oligonucleotide comprises about 7 to about 30 nucleotides.
5. A method according to any one of claims 1 to 4, wherein said oligonucleotide is comprised substantially of dG and/or dT nucleotides.
6. A method according to claim 5, wherein the oligonucleotide is selected from the group:

5'-GGGTTGG-3' (Seq ID No 2),

5'-GGGTTGGTTGTGGGT-3' (Seq ID No 4),

5'-GGGTTGGTTGTGGGTGGGTTGGTTGTGGGT-3' (Seq ID No 6),

5'-GGGGTCGGGGTCGGGGTCGGGGTCGGGGTC-5' (Seq ID No 10).

7. A method according to claim 1, wherein said glycosaminoglycan-degrading enzyme is an endoglycosidase.

8. A method according to claim 7, wherein said endoglycosidase is a heparanase or heparitinase enzyme that acts upon heparin and/or heparan sulphate.
9. A method according to claim 8, wherein said endoglycosidase is a mammalian heparanase or a bacterial heparinase.
10. A method according to claim 9 wherein said mammalian heparanase enzyme is a heparanase produced by platelets, macrophages, neutrophils, leukocytes, endothelial cells, smooth muscle cells, human carcinoma or tumour cells.
11. A method according to claim 9, wherein said bacterial heparanase is derived from *Flavobacterium heparinum*.
12. A method according to claim 10, wherein said human carcinoma or tumour cells are selected from melanoma cells, carcinoma cells, fibrosarcoma cells, lymphoma cells, myeloid leukemia cells, and mastocytoma cells.
13. A method according to claim 10, wherein said oligonucleotide additionally inhibits smooth muscle cell activation, proliferation or migration.
14. A method for the treatment of disease associated with glycosaminoglycan-degrading enzymes which comprises administering to a subject in need of such treatment a therapeutically effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier.
15. A method according to claim 14 which is a method for the treatment of cancer, inflammation, autoimmune disorders, infection caused by pathogenic organisms or cardiovascular disease.

16. A method according to claim 15 which is a method for the treatment of vascular hyperplasia, restenosis or atherosclerosis.
17. A method according to any one of claims 14 to 16, wherein said oligonucleotide is an oligonucleotide as defined in any one of claims 2 to 6.
18. A method for the suppression of smooth muscle cell activation, migration and proliferation which comprises contacting said cells with an oligonucleotide according to any one of claims 1 to 6.
19. A method for the treatment vascular hyperplasia or restenosis which comprises administering to a subject in need of such treatment a therapeutically effective amount of an oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, optionally in association with a pharmaceutically acceptable carrier.
20. A method according to claim 19 wherein said oligonucleotide is an oligonucleotide as defined in any of claims 2 to 6.
21. The use of an oligonucleotide for the manufacture of a medicament for the treatment of disease associated with glycosaminoglycan-degrading enzymes, characterized in that at least one backbone linkage between adjacent nucleosides of the oligonucleotide is substituted with one or more sulphur atoms.
22. The use according to claim 21, wherein said disease is selected from cancer, inflammation, autoimmune disorders, infection caused by pathogenic organisms, and cardiovascular disease.

23. The use according to claim 22, wherein said cardiovascular disease is vascular hyperplasia, restenosis or atherosclerosis.
24. Use of an oligonucleotide for the manufacture of a medicament for the treatment of vascular hyperplasia or restenosis, characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms.
25. The use according to any one of claims 21 to 24, wherein said oligonucleotide is an oligonucleotide as defined in any one of claims 2 to 6.
26. An agent comprising an oligonucleotide for the treatment of disease associated with glycosaminoglycan-degrading enzymes, wherein said oligonucleotide comprises at least one backbone linkage between adjacent nucleosides which is substituted with one or more sulphur atoms.
27. An agent according to claim 26, wherein said disease is selected from cancer, inflammation, autoimmune disorders, infection caused by pathogenic organisms, and cardiovascular disease.
28. An agent according to claim 27, wherein said cardiovascular disease is vascular hyperplasia, restenosis or atherosclerosis.
29. An agent comprising an oligonucleotide for the treatment of vascular hyperplasia, restenosis or atherosclerosis, wherein said oligonucleotide comprises at least one backbone linkage between adjacent nucleosides which is substituted with one or more sulphur atoms.

30. An agent according to any one of claims 26 to 29, wherein said oligonucleotide is an oligonucleotide as defined in any one of claims 2 to 6.
31. An oligonucleotide characterized in that at least one backbone linkage between adjacent nucleosides in the oligonucleotide is substituted with one or more sulphur atoms, and further characterized in that said oligonucleotide is an inhibitor of a glycosaminoglycan-degrading enzyme.
32. An oligonucleotide according to claim 31, wherein said oligonucleotide wholly or partially contains phosphorothioate linkages between adjacent nucleotides.
33. An oligonucleotide according to claim 31, wherein said oligonucleotide wholly or partially contains phosphorodithioate linkages between adjacent nucleotides.
34. An oligonucleotide according to claim 31, wherein said glycosaminoglycan-degrading enzyme is an endoglycosidase.
35. An oligonucleotide according to claim 34, wherein said endoglycosidase is a heparanase or heparitinase enzyme that acts upon heparin and/or heparan sulphate.
36. An oligonucleotide according to any one of claims 31 to 35 wherein said oligonucleotide comprises about 7 to about 30 nucleotides.
37. An oligonucleotide according to any one of claims 31 to 36 wherein said oligonucleotide is comprised solely or primarily of dG and/or dT nucleotides.

38. An oligonucleotide according to claim 31, wherein the backbone linkages between adjacent nucleosides of the oligonucleotide wholly comprise phosphorothioate linkages, and said oligonucleotide is selected from the group:

5'-GGGTTGG-3' (Seq ID No 2),

5'-GGGTTGGTTGTGGGT-3' (Seq ID No 4),

5'-GGGTTGGTTGTGGGTGGGTGGTTGTGGGT-3' (Seq ID No 6),

5'-GGGGTCGGGGTCGGGGTCGGGGTCGGGGTC-5' (Seq ID No 10).



# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 95/00600

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup>: C12N 9/24, 9/88; C07H 21/04; A61K 31/73

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

WPAT: CASM: keywords as attached (a)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN nucleotide sequence search (see sequence attached (b))

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Biol. Chem, vol.266 no.15, pages 9661-9666 (25 May 1991) Nakajima <i>et al.</i> : "Sumarin, a patent inhibitor of melanoma heparanase and invasion" (whole document)	1-38
A	WO, A, 94/08053 (ISIS Pharmaceuticals, Inc.) published 14 April 1994 (see whole document, in particular page 14, lines 2-31; page 24, line 23- page 28, line 16)	1-38
A	EP, A, 463712 (University Patents, Inc.) published 2 January 1992 (see whole document, in particular lines 16-26).	1-38

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Further documents are listed in the continuation of Box C

☒

See patent family annex

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

6 January 1995

Date of mailing of the international search report

19 DECEMBER 1995

Name and mailing address of the ISA/AU  
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**INTERNATIONAL SEARCH REPORT**

International Application No.

**PCT/AU 95/00600**

<b>C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
A	WO, A, 91/15500 (E.I. du Pont du Nemours and Company) published 17 October 1991 (see page 6, line 30-page 7, line 9)	1-38
A	EP, A, 342544 (Taito Co., Ltd) published 23 November 1989 (see page 2, lines 16-24)	1-38

**INTERNATIONAL SEARCH REPORT**

International Application No.

PCT/AU 95/00600

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**Box B. FIELDS SEARCHED (continued)****Keywords**

(a)

WPAT:JAPIO:USPM:C12N 9/24 or C12N 9/36 or C12N 9/38 or C12N 9/40 or C12N 9/42 or C12N 9/44 or C12N 9/88 or C12N 9/99 and inhibit: or inactiv: or decreas: or reduc: and nucleotide# or oligonucleotide or sulphur or sulfur or phosphorothioate or phosphodithioate

**CASM:**

search 1: enzyme classes 3.2 or 4.2.2 and modified link/NTE

search 2: enzyme classes (as above) and sulphur or sulfur or phosphothioate or phosphodithio.

(b)

STN: nucleotide sequence search:

GGGTTGG

GGGTTGGTTGTGGGT

GGGTTGGTTGTGGGTGGGTTGGTTGTGGGT

GGGGTCGGGGTCGGGGTCGGGGTCGGGGTC

**INTERNATIONAL SEARCH REPORT****Information on patent family members**

International Application No.

**PCT/AU 95/00600**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	94/08053	EP	672193	FI	951467	IL	107150
		HU	9500911	NO	951191		
EP	463712	AU	71057/91	CA	2036287	JP	6009682
		US	5278302	US	5453496	AU	37392/82
		EP	378615	ES	2015665	IL	90359
		IL	110600	JP	3501128	WO	89/11486
		ZA	89/03840	US	5218103	AU	54182/90
		AU	66036/90	WO	90/12022	WO	91/04983
WO	95/15500	AT	121416	AU	74581/91	CA	2079777
		DE	69109100	EP	517796	ES	2071989
		HK	1094/95	IL	97804	JP	5506014
		NZ	237723				
EP	342544	DE	68910577	JP	1287031	US	5053398
END OF ANNEX							